REMARKS

In the Official Action dated July 14, 2004, claims 47-55 are pending. Claims 49 and 50 are withdrawn from further consideration as drawn to non-elected subject matter. Claims 47, 48 and 51-55, drawn to plant delta-6 desaturases, are under examination. Claims 54 and 55 are objected to under 37 C.F.R. § 1.75(c) because of improper multiple dependencies. Claims 47, 48 and 51 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Claims 47-48 and 51-55 are rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to satisfy the written description requirement. Claims 47, 48, and 51-55 are further rejected under 35 U.S.C. § 112, first paragraph, for allegedly lacking enabling support in the specification. Additionally, the Examiner has indicated that the application filed under former 37 CFR § 1.60 lacks the necessary reference to the parent application.

This Response addresses each of the Examiner's rejections and objections. Applicant therefore respectfully submits that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

The Examiner has indicated that the instant application lacks the necessary reference to the parent application. The Examiner has required Applicant to include in the specification a cross-reference to the parent application. The Examiner has also required Applicant to include in the cross-reference section the current status of all referenced non- provisional parent applications.

Applicant respectfully submits that the Preliminary Amendment, filed on December 21, 2001, added a reference to the parent application, U.S. Serial No. 08/934,254 filed on September 19, 1997. Additionally, the Preliminary Amendment included references to all non-provisional

parent applications and the status thereof. Applicant has now amended the specification by adding the patent number issued from the parent application. Therefore, Applicant respectfully submits that the objection to the specification is overcome. Withdrawal of the objection is respectfully requested.

The Examiner has objected to claims 54 and 55 under 37 C.F.R. § 1.75(c) allegedly because of improper multiple dependencies. Claims 54 and 55 both depend on claims 47 and 51.

Applicant has amended claims 54 and 55 to only depend on claim 47. Furthermore, Applicant has added new claims 56 and 57 that only depend on claim 51. No new matter has been added. In view of the foregoing, withdrawal of the objection is therefore respectfully requested.

The Examiner has rejected claims 47, 48 and 51 under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Specifically, the Examiner alleges that claim 47 is indefinite because the recitation "delata". The Examiner suggests amending claim 47 to read "delta". Further, the Examiner states that claim 48 is indefinite because the recitation, "wherein said isolated nucleic acid is a plant delta-6 desaturase," implies that the DNA is a protein. The Examiner suggests amending claim 48 to read that the nucleic acid encodes the delta-6 desaturase. Moreover, the Examiner alleges that claim 51 is indefinite because of the recitation "an isolated nucleic acid'. The Examiner states that claim 51 should recite "the isolate nucleic acid".

Applicant has amended claims 47, 48 and 51, as suggested by the Examiner. Therefore, Applicant respectfully requests that the Examiner withdraw the rejection of claims 47, 48 and 51 under § 112, second paragraph.

The Examiner has rejected claims 47-48 and 51-55 under 35 U.S.C. § 112, first paragraph, for allegedly failing to satisfy the written description requirement. Specifically, the Examiner contends that the specification does not describe the subject matter in a manner that conveys to those skilled in the art that Applicant had possession of the claimed invention at the time the application was filed. The Examiner alleges that the specification does not describe any structural feature of the claimed nucleic acid in order to define a genus. The Examiner also alleges that it is not clear in the specification what nucleic acid sequence or combination of nucleic acid sequences is required to encode a delta-6 desaturase.

Applicant respectfully submits that the specification describes not only the *Borage* and *Evening Primrose* delta-6 desaturase DNAs, but also a gain of function assay for characterizing the enzymatic activity of a plant delta-6 desaturase DNA (the conversion of linolenic acid (LA) to gamma linolenic acid (GLA)). See, for example, page 7, lines 23-28, page 10, lines 25-29, and page 11, lines 1-4, 25-28 of the specification. Furthermore, contrary to the Examiner's allegation, the specification teaches characteristics of a delta-6 desaturase encoded by the claimed nucleic acid. For example, the specification teaches structural motifs in the *Borage* delta-6 desaturase, namely, the Lipid Box, the Metal Box 1, and the Metal Box 2. See page 39, lines 12, 13, and Table 3 of the specification. The specification further describes, the three identical motifs, renamed as "histidine rich" motifs, in the *Evening Primrose* delta-6 desaturase. See , page 48, lines 26-28, page 49, lines 2-12. The specification also teaches, at page 48, lines 26, 27, that the three "histidine rich" motifs are common structural features present in all plant delta-6 desaturases. In addition, the specification discloses, in page 11, lines 27, 28 and page 12, lines 1-11, that isolated DNA sequences encoding plant delta-6 desaturases could be

characterized by molecular hybridization to the *Borage* or *Evening Primrose* delta-6 desaturase DNA.

Applicant respectfully submits that the gain of function assay, the three motifs of the *Borage* delta-6 desaturase DNA, and the hybridization methods, were all disclosed by Applicant at least as early as December 30, 1994 in U.S. Application Serial No. 08/366,779.

In *Enzo Biochem v. Gen-probe, Inc.*, 323 F.3d 956, at 964 (Fed. Cir. 2002), the Court addressed the factors in determining whether the written description is met.

> "It is not correct, however, that all functional descriptions of genetic material fail to meet the written description requirement. . . the written description requirement can be met by 'show[ing] that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics . . . *i.e.,* complete or partial structure, other physical and/or chemical properties, *functional characteristics when coupled with a known or disclosed correlation between function and structure,* or some combination of such characteristics.'"

See *Enzo Biochem* at 964 (emphasis in original).

The court in *Enzo Biochem* also stated that the written description requirement is met if one skilled in the art finds the generically claimed sequences, described on the basis of the disclosure of the hybridization function and an accessible structure, consistent with the United States Patent and Trademark Office (USPTO) Guidelines. According to the USPTO Guidelines, claims by functional language are permissible when the claimed material hybridizes to a disclosed substrate. See *Enzo Biochem* at 968.

To delineate a preferred embodiment of the present invention, Applicant has further added claims 58-59, drawn to a plant delta-6 desaturase nucleotide sequence that hybridizes to the complement of the *Borage* delta-6 desaturase DNA or to DNAs encoding polypeptide fragments which correspond to portions of the *Borage* delta-6 desaturase amino acid sequence.

Support for the subject matter of claims 58 -59 is found in the specification in page 11, lines 25 – 28, page 12, lines 1-7, Example 8 at page 37, lines 11-22 and Figure 5B.

In view of the foregoing, Applicant respectfully submits that the specification discloses sufficient detailed and relevant identifying features of the claimed molecules, e.g., the gain of function assay, the three "histidine rich" motifs, and the hybridization features, such that those skilled in the art would understand that Applicant had possession of a DNA encoding a plant delta-6 desaturase at the time the application was filed. Thus, Applicant respectfully submits that the present application complies with the written description requirement under 35 U.S.C. § 112, first paragraph. Applicant respectfully requests that the Examiner withdraw the rejection of claims 47-48 and 51-55 based on the written description requirement.

The Examiner has also rejected claims 47, 48 and 51-55 under 35 U.S.C. § 112, first paragraph, allegedly because the specification does not provide enablement for delta-6 desaturases from all plant species.

Specifically, the Examiner contends that the specification has only disclosed nucleic acids encoding delta-6 desaturases from two plant species, *Evening Primrose* and *Borage*. The Examiner further alleges that the specification does not teach what sequences these nucleic acids have in common that identify them as delta-6 desaturase genes, or how to identify other nucleic acids that encode delta-6 desaturases.

Applicant respectfully submits that the specification discloses specific structural characteristics of plant delta-6 desaturases, e.g., the "histidine rich" motifs. The specification also discloses, *inter alia*, a gain of function assay and hybridization conditions for identifying homologous sequences. Therefore, the specification provides sufficient guidance for those

skilled in the art to identify nucleic acids encoding plant delta-6 desaturases and to use such nucleic acids for modifying lipid metabolism in a plant.

The Examiner refers to WO 99/2711, citing that delta-6 desaturation "is an unusual modification in higher plants occurring only in a small number of species such as borage, evening primrose and redcurrant". In addition, the Examiner indicates that sequence homology is not sufficient to predict function of encoded sequences. In support of his position, the Examiner has cited Doerks et al., *TIG*, 14 6:248-250 (June 1998), Smith et al., *Nature Biotechnology*, 15:1222-1223 (November 1997), Brenner et al., *TIG*, 15 4:132-133 (April 1999), and Borks et al. *TIG*, 12 10:425-427 (October 1996), Van de Loo et al., *Proc. Natl. Acad. Sci.* 92:6743-6747 (July 1995) and Broun et al., *Science*, 282:1315-1317.

Applicant respectfully submits that in addition to specific plant nucleic acid sequences, the specification also describes three common structural motifs designated as "Lipid Box, Metal Box 1 and Metal Box 2" or "histidine rich" motifs. See page 48, lines 26–28, page 49, lines 2-3, Figure 5B and Figure 10. Moreover, the specification teaches that delta-6 desaturases can be characterized by a gain of function assay. See page 14, lines 3-5. Furthermore, the specification discloses the isolation of DNA encoding the *Primrose* delta-6 desaturase using primers derived from the DNA sequence encoding the *Borage* delta-6 desaturase. See page 13, lines 1-6 and in Example 15. Thus, the instant disclosure has overcome the pitfalls of a general sequence analysis employed in the cited references, which is based solely in sequence homology. Based on the distinguishing features of a plant delta-6 desaturase disclosed in the specification, those skilled in the art would be able to determine whether an isolated nucleic acid sequence encodes a plant delta-6 desaturase, without undue experimentation.

As evidence that identification of additional plant delta-6 desaturases genes would not be undue, Applicant respectfully submits the following publications: Sperling et al., *Eur. J. Biochem.*, 267:3801-3811 (2000), Girke et al., *The Plant Journal*, 15:39-48 (1998) and Garcia-Maroto, *Lipids*, 37:417-426 (2002).

In Sperling et al., the delta-6 desaturase from the plant, *Ceratodon purpureus,* was identified and characterized using primers derived from the sequence fragments encoding "histidine rich" domains and a yeast gain of function assay.

In Girke et al., the delta-6 desaturase cDNA of *Physcomitrella patens* was identified using primers derived from sequences containing similar "histidine rich" motifs, and a function assay that included the profiling of fatty acids by gas liquid chromatography.

In Garcia-Maroto, the genes encoding the delta-6 desaturase of two plants, *Echium gentianoides and Echium pitardii*, were identified and characterized using primers derived from sequences containing similar "histidine rich" motifs.

The Examiner also cites De Luca, *AgBiotech News and Information*, 5: 225N-229N (1993), alleging that transforming plants with genes encoding enzymes is highly unpredictable and that in many occasions the goals are impossible to achieve.

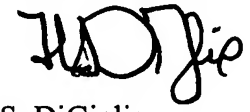
Applicant respectfully submits that De Luca refers to unsuccessful transformations of plants with genes encoding proteins other plant delta-6 desaturases. Applicant draws the Examiner's attention to the teaching in De Luca that shows that the alteration of lipid metabolic pathways using enzymes involved in fatty-acid biosynthesis, is an efficient type of alteration that is devoid of unpredictability. See De Luca at page 228N, first column, second, third and fourth paragraphs. Further, Applicant submits that the instant specification, in Examples 10-13 and Example 16, demonstrates successful transformation of tobacco and carrot with the *Borage* and

Primrose delta-6 desaturase genes. Based on the plant transfection methods and techniques provided in the specification, those skilled in the art would be able to modify the lipid composition of a plant with a delta-6 desaturase gene without undue experimentation. In this connection, it is observed that in Garcia-Maroto (*supra*), similar to what is described in the specification, tobacco was transformed with the isolated delta-6 desaturase genes and the resulting fatty acid profiles were analyzed using gas liquid chromatography.

In view of the foregoing, Applicant respectfully submits those skilled in the art would be able to isolate a plant delta-6 desaturase gene, as presently claimed, without undue experimentation. Withdrawal of the rejection of claims 47, 48 and 51-55 based on the enablement requirement, is therefore respectfully requested.

In view of foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,



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A bifunctional Δ^6-fatty acyl acetylenase/desaturase from the moss *Ceratodon purpureus*

A new member of the cytochrome b_5 superfamily

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Many plant genes have been cloned that encode regioselective desaturases catalyzing the formation of *cis*-unsaturated fatty acids. However, very few genes have been cloned that encode enzymes catalyzing the formation of the functional groups found in unusual fatty acids (e.g. hydroxy, epoxy or acetylenic fatty acids). Here, we describe the characterization of an acetylenase from the moss *Ceratodon purpureus* with a regioselectivity differing from the previously described Δ^{12}-acetylenase. The gene encoding this protein, together with a Δ^6-desaturase, was cloned by a PCR-based approach with primers derived from conserved regions in Δ^5-, Δ^6-fatty-acid desaturases and Δ^8-sphingolipid desaturases. The proteins that are encoded by the two cloned cDNAs are likely to consist of a N-terminal extension of unknown function, a cytochrome b_5-domain, and a C-terminal domain that is similar to acyl lipid desaturases with characteristic histidine boxes. The proteins were highly homologous in sequence to the Δ^6-desaturase from the moss *Physcomitrella patens*. When these two cDNAs were expressed in *Saccharomyces cerevisiae*, both transgenic yeast cultures desaturated Δ^9-unsaturated C16- and C18-fatty acids by inserting an additional Δ^6-*cis*-double bond. One of these transgenic yeast clones was also able to introduce a Δ^6-triple bond into γ-linolenic and stearidonic acid. This resulted in the formation of 9,12,15-(*Z*,*Z*,*Z*)-octadecatrien-6-ynoic acid, the main fatty acid found in *C. pupureus*. These results demonstrate that the Δ^6-acetylenase from *C. pupureus* is a bifunctional enzyme, which can introduce a Δ^6-*cis*-double bond into 9,12,(15)-C18-polyenoic acids as well as converting a Δ^6-*cis*-double bond to a Δ^6-triple bond.

Keywords: *Ceratodon purpureus*; cytochrome b_5-fusion protein; Δ^6-fatty acid acetylenase; Δ^6-fatty acid desaturase; *Saccharomyces cerevisiae*.

In addition to the common fatty acids, plants produce a variety of unusual fatty acids such as hydroxy, epoxy or acetylenic fatty acids [1]. Unsaturated fatty acids are formed by desaturases, which catalyze the introduction of double bonds into preformed acyl chains by removal of a pair of hydrogens, concomitant oxidation of an electron donor and reduction of O_2 [2,3]. The cloning and sequencing of desaturases from different organisms identified two distinct groups, soluble acyl-ACP desaturases and membrane-bound desaturases, which differ in their consensus motifs [2]. The membrane-bound desaturases contain three characteristic histidine motifs, which are believed to coordinate a di-iron cluster in the active site. The comparison of amino-acid sequences has revealed, that these desaturases may be grouped according to differing regioselectivity, which presently spans from C5–C15 [3]. Genes that encode enzymes closely related to the microsomal Δ^{12}-desaturases, have been cloned and found to catalyse exotic modifications at or around the Δ12 position of the acyl chain, reactions such as hydroxylation, epoxidation and acetylenation. A Δ^{12}-hydroxylase gene, that encodes an enzyme responsible for the production of 12-hydroxy-9-(Z)-octadecenoic acid (ricinoleic acid) from oleic acid, was identified by mass sequencing of cDNAs from *Ricinus communis* [4]. On the basis of homology, the gene encoding the *Lesquerella fendleri* oleate Δ^{12}-hydroxylase was subsequently isolated. Unlike the enzyme from *R. communis*, the *L. fendleri* had both hydroxylase and desaturase activities [5]. Site directed mutagenesis of the *Lesquerella* oleate Δ^{12}-desaturase has shown that as few as four amino-acid substitutions can convert a strict desaturase to a bifunctional hydroxylase [6]. In *Crepis rubra* and *Crepis alpina* linoleic acid and possibly oleic acid are substrates for the synthesis of 9-(Z)-octadecen-12-ynoic acid (crepenynic acid) [7,8]. In the moss *Ceratodon pupureus* 9,12-(Z,Z)-octadecadien-6-ynoic and 9,12,15-(Z,Z,Z)-octadecatrien-6-ynoic acid are formed by the generation of an acetylenic bond at the pre-existing Δ^6-*cis*-double bond of a C_{18}-fatty acid [9]. Recently, a Δ^{12}-acetylenase from *Crepis alpina* and a Δ^{12}-epoxidase from *Crepis palaestina* were cloned and functionally expressed in yeast and *Arabidopsis thaliana* [8]. Both of these enzymes were similar in

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Abbreviations: DEPT, distortionless enhancement by polarization transfer; DMOX, 4,4-dimethyloxazoline; FAME, fatty-acid methyl ester; GC-MS, gas-liquid chromatography mass spectrometry; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; mu, mass units; Cmdum, minimal-dropout-uracil medium.

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biochemical properties and sequence to the microsomal Δ^{12}-desaturases. Therefore, at least four reactions (desaturation, hydroxylation, epoxidation and acetylenation) have been shown to be catalyzed by closely related desaturase-like enzymes [8]. More recently, it has been shown that even the conversion of linoleic acid (9*Z*,12*Z*-18:2) to conjugated octadecatrienoic acids such as α-eleostearic acid (9*Z*,11*E*,13*E*-18:3) and calendic acid (8*E*,10*E*,12*Z*-18:3) is catalyzed by Δ^{12}-desaturase-like enzymes [10,11]., Regioselectivity appears to place greater constraints on the sequence of fatty-acid desaturase and desaturase-related enzymes than actual reaction outcome (i.e. formation of *cis*- or *trans*-double bonds, triple bonds, epoxy or hydroxy groups) [3]. A comparison of sequences from cloned desaturases has revealed, that Δ^5-, Δ^6- and Δ^8-desaturases all fall into the same regioselectivity group [3,12]. We speculated, that an enzyme that could catalyze acetylenation at C6 is likely to be related to the Δ^6-desaturases, of which most are cytochrome b_5-fusion proteins [13]. This predicted similarity was used to clone an acetylenase with a regioselectivity different from the Δ^{12} position. Here we report the PCR-based isolation of a bifunctional Δ^6-acetylenase/desaturase and a Δ^6-fatty-acid desaturase from the moss *C. pupureus*. Their expression in *Saccharomyces cerevisiae* and feeding experiments with different fatty-acid substrates, has led to their functional identification.

MATERIALS AND METHODS

Single-stranded cDNA and cDNA library

Poly(A)$^+$ RNA was isolated from about 400 mg of *C. purpureus* protonemata (a gift of P. Beutelmann, Institut für Allgemeine Botanik, Universität Mainz, Germany) with a Quickprep Micro (Amersham Pharmacia Biotech Inc.) according to the manufacturer's instructions. The mRNA was precipitiated and used to synthesize first-strand cDNA with oligo $dT_{(12-18)}$ and MMLV reverse transcriptase (Gibco BRL). This cDNA was used directly for PCR. The moss λ-ZAP cDNA library was a generous gift from F. Thümmler (Botanisches Institut, Universität München, Germany) and was prepared as follows: total RNA was extracted from light-grown and 7-day dark adapted *C. pupureus* wt3 protonemata and from a 1:1 mixture of this RNA poly(A)$^+$ RNA was prepared. cDNA, prepared from this poly(A)$^+$ RNA, was used to construct a directional cDNA library with *Eco*RI/*Xho*I-cut uni-ZAP XR vector arms (Stratagene) [14].

PCR amplification with degenerated primers

For a PCR-based cloning, single-stranded cDNA was used as a template. One PCR fragment of 557 bp (Cer3) was amplified with the degenerated forward primer A 5′-TGGTGGAA(A/G)TGGA(A/C)ICA(C/T)AA-3′ and the reverse primer C 5′-AT(A/T/G/C)T(T/G)(A/T/G/C)GG(A/G)AA(A/T/G/C)A(A/G)(A/G)TG(A/G)TG-3′. These primers were derived from the amino-acid sequence WWKW(N/T/K)H(N/K), and (I/M)(H/Q/N)PF(L/F)HH, respectively. A second 560 bp fragment (Cer1) was amplified with another forward primer B 5′-(T/G)GITGGA-A(A/G)(T/G)(G/A)I(A/C)AICA(C/T)AA-3′ derived from the amino-acid sequence (G/W)WK(E/D/W)(N/Q/K)H(N/K) and the reverse primer C, mentioned above. The PCR amplification was carried out using the following program: 10 min denaturation at 94 °C, brake for 'hot start' with *Taq* DNA polymerase at 72 °C, followed by 32 cycles of 20 s at 94 °C, 1 min at 45 °C, 1 min at 72 °C and terminated by 10 min extension at 72 °C. The PCR fragments of the expected length (500–600 bp) were ligated into pGEM-T (Promega) and transformed into *Escherichia coli* Xl1blue MRF′ Kan (Stratagene). Plasmid-DNA mini-preparations [15] p1 and p3 were sequenced by the dideoxy chain termination method using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Weiterstadt).

cDNA library screening for full length clones

The inserts from the plasmids p1 and p3 were excised with *Nco*I and *Pst*I and gel purified. These were radiolabelled by random priming with [α-^{32}P]dCTP and used together to screen the λ ZAP cDNA library from *C. pupureus* wt3 protonemata by hybridization. Filters were hybridized at 55 °C and washed at 55 °C with 1 × NaCl/Cit and 0.1% SDS. Individual plaques, with sequences that corresponded to either p1 or p3, were further identified, and distinguished from each other, by PCR analysis with the primer pairs 5′-CGAATGAGTGCGAC-GAAC-3′ and 5′-AATAACCTGGGCTCTCAC-3′ (for the p1 sequence), and 5′-ATGAGGATATTGATACTCTC-3′ and 5′-GCAATCTGGGCATTCACG-3′ (for the p3 sequence). To identify full length cDNA clones individual plaques were analyzed by PCR with the T7 and T3 primer to identify clones containing the longest inserts. Two λ clones, one representing the 1p sequence and the other the 3p sequence, were investigated further. These had inserts of ≈2.2 kb in size. The corresponding bacteriophages were isolated by precipitation with poly(ethylene glycol) and from these DNA was isolated. The inserts were excised with *Eco*RI and *Kpn*I and cloned into the *Eco*RI and *Kpn*I sites of pUC19, resulting in plasmids pCer1 and pCer3 and sequenced on both strands, which corresponded to the sequences 1p and 3p, respectively.

Expression in *S. cerevisiae*

The ORFs of pCer1 and pCer3 were cloned behind the galactose-inducible promotor *GAL 1* of the yeast expression vector pYES2 (Invitrogen). In order to achieve this, a new 5′ *Kpn*I (upstream of the deduced translation start) and a 3′ *Eco*RI site (downstream the stop codon) were introduced into the inserts of pCer1 and pCer3 by PCR. These modified inserts were digested with *Kpn*I and *Eco*RI and ligated into the corresponding restriction sites of pYES2 to yield pYCer1 and pYCer3. Their sequences were verified by DNA sequencing, as described above. These plasmids and the empty vector pYES2 were transformed into *S. cerevisiae* strain INVSc1 (Invitrogen) using the polyethylenglycol method [16].

Transformed cells were grown in complete minimal-dropout-uracil medium (CMdum) supplemented with 2% raffinose as the only carbon source [17] and 1% Tergitol NP-40 [(w/v); Sigma] for the solubilization of fatty acids (Sigma) [18]. For expression experiments, test cultures in CMdum medium supplemented with 0.003% of the corresponding fatty acid [(w/v); stock solution solubilized in 5% Tergitol] were aerobically grown to logarithmic phase, then induced with 1.8% galactose [(w/v); final concentration] and finally grown to saturation for 24 h at 30 °C.

Gas-liquid chromatography mass spectrometry (GC-MS) analysis of fatty acids

C. pupureus and Dicranum scoparium cultures (generous gifts from E. Hartmann, Institut für Biologie, Freie Universität Berlin, Germany and from P. Beutelmann, Institut für Allgemeine Botanik, Universität Mainz, Germany) as well as

cell pellets (≈ 100 mg) from wild-type (pYES2) and transgenic yeast (pYCer1 and pYCer3) were subjected to acid methanolysis and fatty-acid methyl esters (FAME) were obtained, as described before [19]. FAME were analyzed by gas-liquid chromatography on a polar capillary column (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 m, 0.32 mm) with the following temperature program: 1 min at 170 °C, linear temperature gradient (5 °C·min^{-1}) up to 240 °C and 5 min at 240 °C. FAME identities were confirmed by comparison with appropriate reference substances (Sigma).

For the determination of double or triple bond positions, FAME (about 200 μg) were converted into their 4,4-dimethyl-oxazoline (DMOX) derivatives as described [20]. GC-MS of the FAME and DMOX derivatives of the fatty acids was carried out with a Hewlett-Packard Model 5989 equipped with a nonpolar capillary column (HP-MS) using a temperature gradient from 150 °C to 320 °C with a temperature rise of 5 °C·min^{-1}. EI-mass spectra were recorded at 70 eV and CI-mass spectra were obtained at 105 eV with ammonia as reactant gas. The ion-source temperature in both cases was 250 °C. The EI-mass spectra of the DMOX derivatives are shown in Fig. 3, whereas the spectrum of the 18:3A methylester is listed here with *m/z*-values and relative intensities in brackets: M^+(–), 257 (2), 173 (17), 159 (7), 145 (24), 131 (40), 119 (20), 117 (62), 105 (46), 91 (100), 79 (72), 67 (60), 55 (45).

NMR spectroscopy

For the preparation and isolation of pure FAME in mg quantities suitable for NMR analysis *C. pupureus* and *D. scoparium* cultures (500 mg and 300 mg fresh weight, respectively) were subjected to acid methanolysis as described [19]. Both mixtures containing significant amounts of 18:3A of FAME (as detected by GC) were further separated by preparative TLC using silicagel 60 plates (20 × 20 cm, thickness 0.25 mm, Merck) in petroleum ether/dietyl ether (9:1, v/v). FAME and acetylenic acid methyl esters (R_f values 0.3 and 0.18, respectively) were detected by UV light after spraying the plates with 0.2% (w/v) methanolic 8-anilino-naphthalene-1-sulfonic acid. From these plates acetylenic acid methyl esters were eluted with petroleum ether resulting in 1.4 mg of 18:3A methylester (purity 98% as detected by GC) from *C. pupureus* and 1.2 mg 18:3A methylester (98%) from *D. scoparium*.

Both 1D ^{1}H- and 2D homonuclear and H-detected heteronuclear ^{1}H,^{13}C-correlation spectra [heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC)] were recorded on a 600-MHz spectrometer (Bruker Avance DRX-600). ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra were run at 90.6 MHz (Bruker DPX-360). The methyl ester of 18:3A (isolated from *C. pupureus* and *D. scoparium*, as mentioned above) was dissolved in 500 μL $CDCl_3$ (99.96%, Cambridge Isotope Laboratories, Andover, MA, USA) and NMR spectra were recorded at 300 K with internal TMS ($\delta_H = 0.000$) or $CDCl_3$ ($\delta_C = 77.0$) as reference. 1D (^{1}H-, ^{13}C, and DEPT) and 2D homonuclear (^{1}H,^{1}H COSY) and H-detected heteronuclear (^{1}H,^{13}C HMQC, ^{1}H,^{13}C HMBC) experiments were performed using standard Bruker software (XWINNMR, version 2.6) and resulted in the following assignment for 9,12,15-(*Z*,*Z*,*Z*)-octadecatrien-6-ynoic acid (18:3A) methylester: ^{1}H-NMR (600 MHz, $CDCl_3$): δ 0.977 (3H, *t*, J = 7.5 Hz, H-18), 1.517 (2H, *m*, H-4), 1.724 (2H, *t*, J = 7.2, 10.0 Hz, H-3), 2.077 (2H, *m*, H-17), 2.175 (2H, *tt*, J = 3.3 Hz, H-5), 2.328 (2H, *t*, J = 7.5 Hz, H-2), 2.815 (4H, *m*, H-11 and H-14), 2.932 (2H, *m*, H-8), 3.669 (3H, *s*, Me at C-1), 5.27–5.48 (6H, *m*, H-9, H-10, H-12, H-13, H-15, and H-16); ^{13}C-NMR (90.5 MHz, $CDCl_3$): δ 14.2 (C-18), 17.2 (C-8), 18.5 (C-5), 20.6 (C-17), 24.2 (C-3), 25.5 (C-11 and C-14), 28.4 (C-4), 33.6 (C-2), 51.4 (Me at C-1), 78.5 (C-7), 79.5 (C-6), 125.4 (C-9), 126.9 (C-15), 127.4 and 128.8 (C-12 and C-13), 129.2 (C-10), 132.1 (C-16), and 173.9 (C-1).

RESULTS

PCR based cloning

Degenerated primers matching the three conserved histidine regions [2] were deduced from the aligned amino-acid sequences of cloned Δ^5-, Δ^6-acyl lipid desaturases and Δ^8-sphingolipid desaturases (EMBL accession numbers: Z81122, U79010, AJ222980, AF031477, X87143, AJ224160, AJ224161) [3]. Single-stranded cDNA from the moss *C. pupureus* was used as a template for PCR. A number of PCR fragments, that were of the expected length, were cloned and sequenced. Databank searches and alignments with these new sequences revealed that two of the fragments (1p and 3p) had similarities to Δ^5- and Δ^6-acyl lipid desaturases. The deduced amino-acid sequences of these partial sequences had 64% amino-acid identity to each other, and 74% (3p) and 63% (1p) identity to the Δ^6-desaturase from the moss *Physcomitrella patens* [19]. Because sequence 3p (557 bp) was more similar to the *Physcomitrella* enzyme than sequence 1p (560 bp), we assumed that the 3p fragment might be derived from a gene encoding a Δ^6-desaturase and the 1p fragment from a gene encoding a Δ^6-acetylenase of *C. pupureus*.

Isolation of full-length cDNA

To isolate full-length cDNA clones, the inserts from 1p and 3p were isolated, radiolabelled and used to screen a cDNA library [14] of light-grown and 7-day dark adapted protonemata. On a plate containing about 25 000 plaque-forming units, approximately 80 hybridizing plaques were observed. The analysis of individual hybridizing plaques with primers, specific to either the 1p or 3p sequence and vector DNA flanking the cDNA inserts were used to identify clones that contained the longest inserts (see Materials and methods). Two λ clones were taken for further analysis. As we were not able to excise phagmids form the cDNA library, inserts from these clones were subcloned into the plasmid pUC19 to give the plasmids pCer1 and pCer3. The inserts from these plasmids were sequenced on both strands. Plasmid pCer1 contained a cDNA of size 2003 bp, excluding its poly(A) tail, and encoded a putative ORF of 483 amino acids between the nucleotide positions 176–1624 (CPAcet6; Fig. 1). Two in-frame stop codons preceeded CPAcet6, which indicated that the sequence upstream of the initiation codon, deduced for CPAcet6, is likely to be the 5′-untranslated region. The pCer3 cDNA had a length of 2142 bp, but no stop codon was detected in its 5′-untranslated region. The nucleotide sequence between positions 159–1718 encoded an ORF of 520 amino acids, which was designated CPDes6 (Fig. 1).

Comparison with other desaturases

Both the predicted proteins, CpDes6 and CpAcet6 (Fig. 1), were slightly shorter than the Δ^6-desaturase from *Physcomitrella patens* (PpDes6), which has an ORF of 525 amino

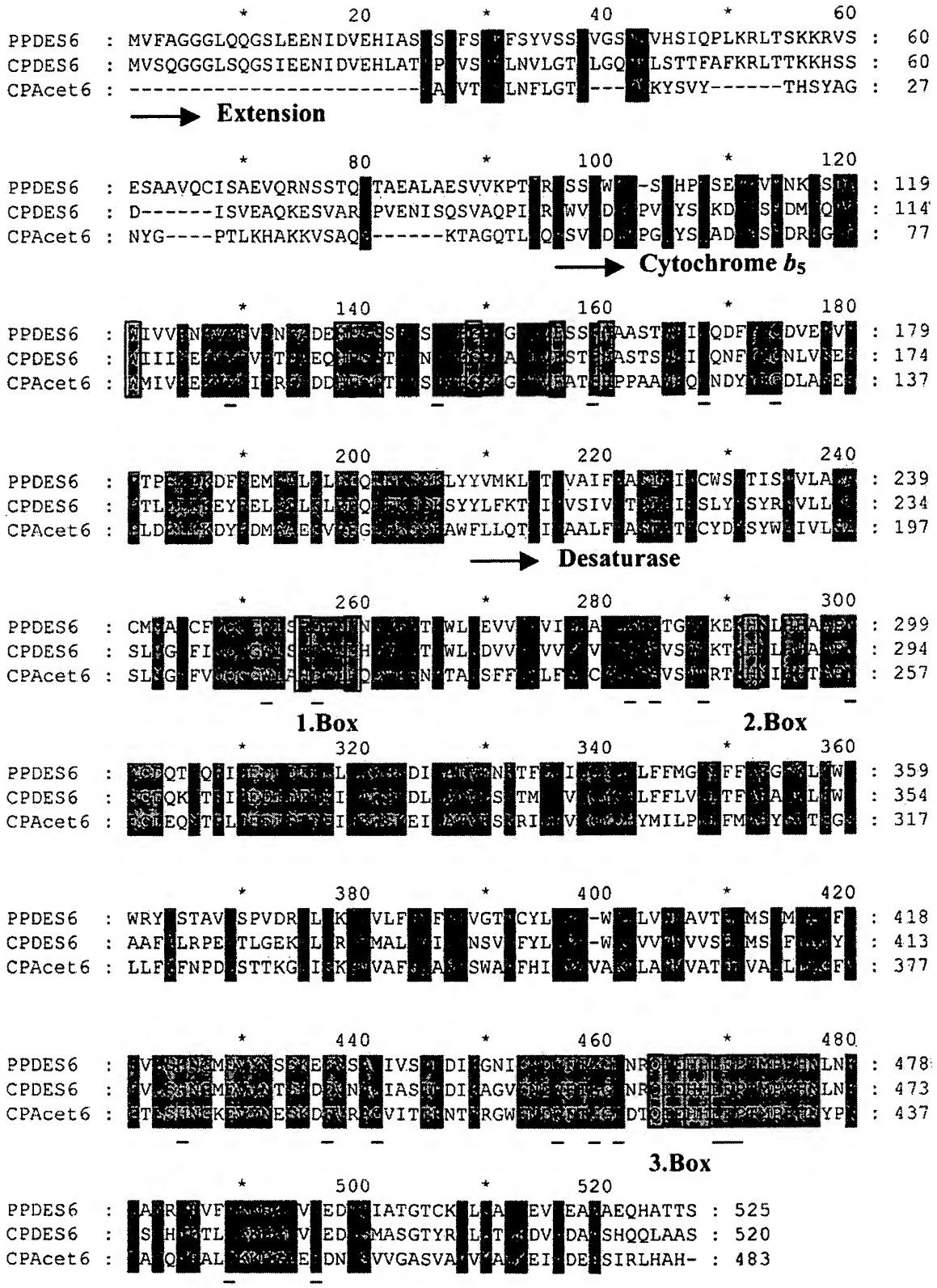

Fig. 1. Amino-acid sequences of the Δ^6-acetylenase (CPAcet6) and the Δ^6-desaturase (CPDES6) from *C. pupureus* and the closely related Δ^6-desaturase (PPDES6) from *P. patens* (AJ222980). For alignment the CLUSTAL X program was used (gap opening of 10, gap extension 0.05) showing invariant residues in grey colour. The approximate beginnings of the three domains are marked by arrows and their putative function. The eight invariant residues characteristic for the cytochrome b_5 superfamily and the three histidine boxes of the desaturase domain are framed. The underlined residues indicate further positions highly conserved in the Δ^6-desaturases from *M. alpina* (AF110510), *B. officinalis* (U79010), *C. elegans* (AF031477) and mammals ([23], AB021980) as well as in the Δ^6-desaturases from cyanobacteria [L11421, X87094], which in contrast to the above mentioned enzymes lack an N-terminal cytochrome b_5-domain.

Table 1. Heterlogous expression of the Δ^6-acetylenase and Δ^6-desaturase from *C. pupureus* in *S. cerevisiae*. Total fatty acids liberated from whole cells transformed with pYES2 (empty vector), pYCer1 (Δ^6-acetylenase) and pYCer3 (Δ^6-desaturase), respectively, were converted into their methyl esters and analysed by GC. Transgenic yeast cells were cultured in selective medium in the absence of fatty acids (–), or in the presence of $18{:}2^{\Delta9,12}$ (18:2), $18{:}3^{\Delta6,9,12}$ (γ-18:3), $18{:}3^{\Delta9,12,15}$ (α-18:3) or $18{:}4^{\Delta6,9,12,15}$ (18:4) to mid logarithmic phase before induction with 2% galactose for 24 h at 30 °C. The proportion of odd numbered saturated and monounsaturated C_{15}- and C_{17}-fatty acids has been summed up and included under 'others'.

	% of total fatty acids														
	pYES2+					pYCer1+					pYCer3+				
Fatty acids	–	18:2	γ-18:3	α-18:3	18:4	–	18:2	γ-18:3	α-18.3	18:4	–	18:2	γ-18:3	α-18:3	18:4
16:0	26.2	24.1	27.8	27.4	32.7	24.2	23.1	26.2	25.7	26.5	26.5	23.3	28.1	29.2	29.6
$16{:}1^{\Delta9}$	41.8	9.6	27.4	27.3	16.1	36.5	13.3	24.7	28.8	21.9	43.8	9.9	25.2	34.0	20.9
$16{:}2^{\Delta6,9}$	–	–	–	–	–	6.9	1.8	3.3	5.3	3.0	1.1	–	0.1	0.8	0.1
18:0	6.5	5.3	6.1	6.1	7.9	6.4	6.1	6.6	6.5	7.1	5.5	5.3	6.3	5.8	5.9
$18{:}1^{\Delta9}$	23.6	4.9	15.1	14.8	11.3	24.9	8.8	15.6	20.0	16.8	21.4	5.3	15.7	14.3	11.5
$18{:}2^{\Delta6,9}$	–	–	–	–	–	0.3	–	0.2	0.3	0.2	0.1	–	–	0.1	–
$18{:}2^{\Delta9,12}$	–	53.9	–	–	–	–	41.9	–	–	–	–	42.3	–	–	–
$18{:}3^{\Delta6,9,12}$	–	–	19.5	–	–	–	0.8	16.1	–	–	–	8.1	21.2	–	–
$18{:}3^{9,12,15}$	–	–	–	22.8	–	–	–	–	10.0	–	–	–	–	11.9	–
$18{:}4^{6,9,12,15}$	–	–	–	–	28.8	–	–	–	1.7	21.3	–	–	–	1.9	30.1
$18{:}2A^{\Delta6a,9,12}$	–	–	–	–	–	–	1.3	4.6	–	–	–	–	–	–	–
$18{:}3A^{\Delta6a,9,12,15}$	–	–	–	–	–	–	–	–	–	2.3	–	–	–	–	–
Others	1.9	2.2	4.1	1.6	3.2	0.8	2.9	2.7	1.7	0.9	1.6	5.8	3.4	2.0	1.9
Σ Desaturation	–	–	–	–	–	7.2	3.9	8.1	7.3	5.5	1.2	8.1	0.1	2.8	0.1

acids [19]. A comparison of cytochrome b_5 fusion desaturase sequences [13] indicated that PpDes6 consists of a hydrophilic cytochrome b_5 domain (from residue 96–208), followed by a C-terminal desaturase domain. The CPDes6 and CPAcet6 sequences were most identical (67% and 52%, respectively) to PpDes6 [19]. They are 33% identical with the Δ^6-desaturase from the fungus *Mortierella alpina* [21] and only 21–24% identical to the Δ^6-desaturase from *Borago officinalis* [22], *Caenorhabditis elegans* [23], mammals ([24], EMBL accession number AB021980) and cyanobacteria [25,26].

The desaturase domains of the two proteins have the three conserved histidine regions characteristic to all membrane-bound desaturases [2]. In addition, the sequence motif QIEHHLFPXMPRXN of the third histidine region is present in these two *C. pupureus* proteins as well as in all the Δ^6-desaturases with an N-terminal cytochrome b_5 fusion. In the Δ^6-desaturases from cyanobacteria [25,26], which lack a cytochrome b_5 domain, the equivalent motif is reduced to QXXHHLFP. The exchange of histidine to glutamine present in the third histidine cluster seems to be a common feature of all Δ^5-, Δ^6- and Δ^8-acyl lipid desaturases and Δ^8-sphingolipid desaturases [13]. Sequence comparisons of the desaturase domain revealed 17 highly conserved amino acids in addition to the seven invariant histidines and the glutamine, which are present in the Δ^6-acetylenase and in all Δ^6-desaturases, known so far (Fig. 1, underlined). Therefore, the signature for the conserved motif of Δ^6-desaturases must be amended to $(W/F)X_{1-2}H(D/E)XXHX_{20-22}GXSX_3WX_3HXX_{1-2}HHX_3NX_{116-134}HX_{11-20}(W/F)X_3QX_{14-15}WX_2GXLX_2QXXHHLFPX_{17}CX_6Y$.

The cytochrome b_5-domain of both proteins contained the eight invariant residues that are characteristic for the cytochrome b_5 superfamily [27]. The deviations in sequence, that are described for the *Helianthus annuus* cytochrome b_5-containing fusion protein [28] were also found in CpDes6 and CpAcet6. A comparison of N-terminal cytochrome b_5 domains revealed five additional amino acids apart from those eight invariant residues mentioned above, which are highly conserved in all Δ^6-fusion desaturases (Fig. 1, underlined). Both CpDes6 and CpAcet6 had putative N-terminal extensions of about 89 and 52 residues, respectively. The function of this sequence, which is also present in the Δ^6-desaturase from the moss *P. patens* (PpDes6; Fig. 1) is unknown [19]. However, if this N-terminal extension codes for a separate domain, the cytochrome b_5 domain of these proteins would represent an internal domain.

The comparison of the amino-acid sequence of the Δ^6-acetylenase with the Δ^6-desaturase sequences from *C. pupureus, P. patens* [19], *M. alpina* [21], *B. officinalis* [22], *C. elegans* [23], mammals ([24], EMBL accession number AB021980) and cyanobacteria [25,26] revealed no strictly conserved residues common to all of these desaturases, but absent in the acetylenase. When the Δ^6-acetylenase/desaturase domain was compared with the two moss Δ^6-desaturase domains (starting at position 209; Fig. 1), 56 amino-acid substitutions were identified. Of these substitutions, 43 were changes between amino acids that were significantly different in chemical property. In contrast to this, only seven strictly conserved amino-acid changes could be identified in the sequences of the Δ^{12}-fatty-acid hydroxylases when compared to the Δ^{12}-fatty-acid desaturases [6]. As few as four to six amino acids were adequate to change the function of these proteins from a desaturase to a hydroxylase or vice versa. The Δ^{12}-acetylenase from *Crepis alpina* [8] deviates from Δ^{12}-desaturases in 29 positions. Only four of these changes are conservative substitutions. Taken together, these data suggest, that a switch from desaturase to acetylenase might involve more extensive changes in sequence than what is required to change a desaturase to a hydroxylase and vice versa. However, it is unclear to what degree these changes are required to facilitate merely substrate recognition (as the acetylenases, in contrast to the desaturases and hydroxylases, presumably recognize

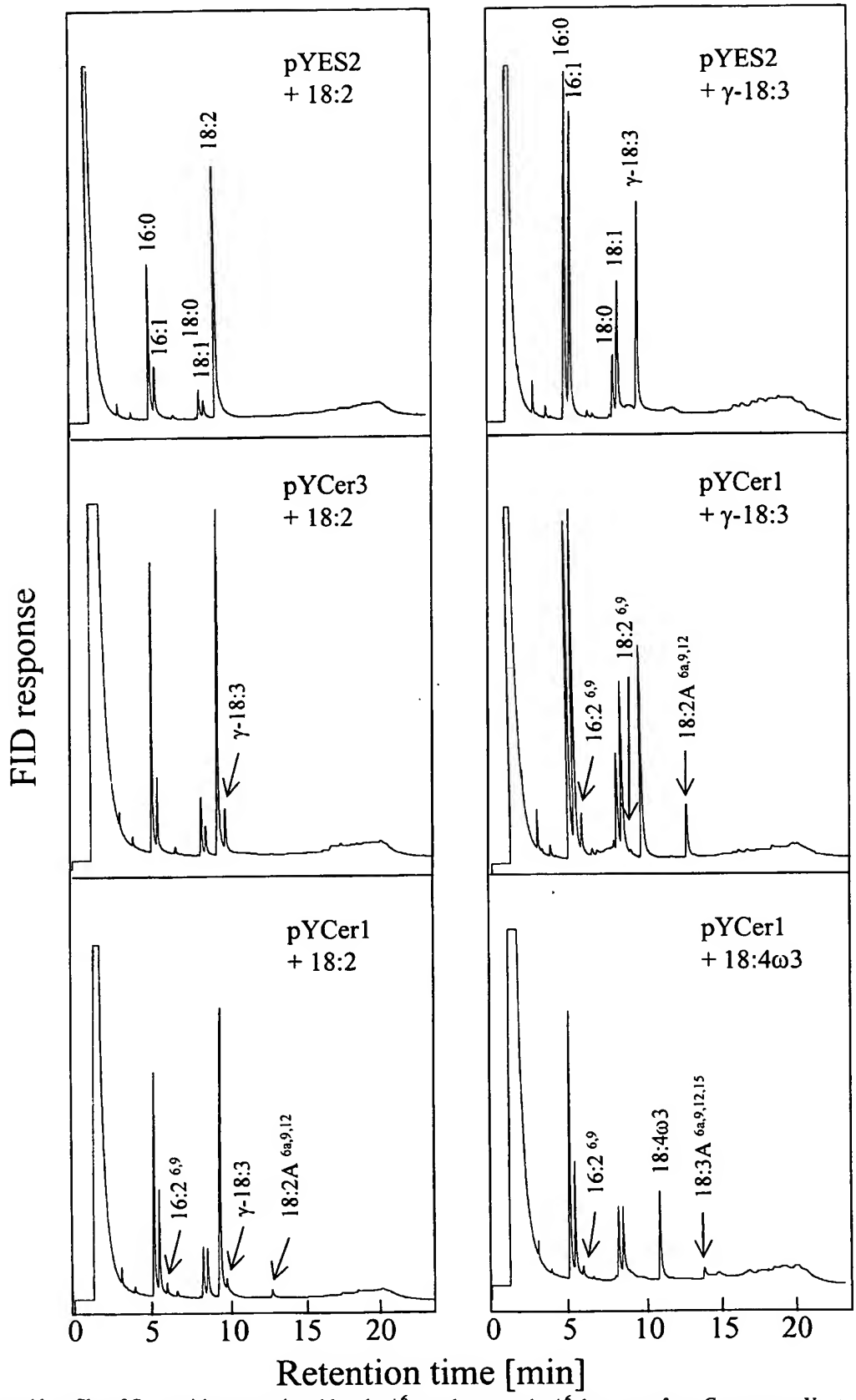

Fig. 2. Fatty-acid profiles of *S. cerevisiae* expressing either the Δ^6-acetylenase or the Δ^6-desaturase from *C. pupureus*. Yeast cultures containing either the empty vector pYES2 (control), pYCer3 or pYCer1 expressing the Δ^6-desaturase or the Δ^6-acetylenase were supplemented with the indicated C18-fatty acids as described under Materials and methods. FAME were prepared from whole cells and analysed by GC. The control strain shows the internal yeast fatty acids (16:0, 16:1, 18:0 and 18:1) and the incorporated fatty-acid substrate (18:2 or γ-18:3). Arrows indicate the desaturation products formed from supplemented $18{:}2^{\Delta 9,12}$ (18:2), $18{:}3^{\Delta 6,9,12}$ (γ-18:3) and $18{:}4^{\Delta 6,9,12,15}$ (18:4ω3), respectively. The acetylenic acids produced are 9,12-(Z,Z)-octadecaden-6-ynoic acid (18:2A) and 9,12,15-(Z,Z,Z)-octadecatrien-6-ynoic acid (18:3A).

substrates with a double bond already present at the Δ^6 or Δ^{12} position) or a differing reaction outcome.

Functional expression in *S. cerevisiae*

For a functional identification, both CpDes6 and CpAcet6 were expressed in *S. cerevisiae*. The cDNAs CpAcet6 and CpDes6 were cloned into a yeast expression vector to give pYCer1 and pYCer3, respectively. These, and the empty vector pYES2 were transformed into *S. cerevisiae* strain INVSc1. Transformed yeast cells were grown to saturation after induction with 1.8% galactose in minimal medium. *S. cerevisiae* contains only saturated and monoenoic fatty-acid substrates, but no di-, tri- or polyenoic fatty acids which may be required for Δ^6-desaturation or Δ^6-acetylenation. Therefore, the medium in which the yeast cells were grown was supplemented with linoleic ($18{:}2^{\Delta 9,12}$), α-linolenic ($18{:}3^{\Delta 9,12,15}$), γ-linolenic ($18{:}3^{\Delta 6,9,12}$) and stearidonic acid ($18{:}4^{\Delta 6,9,12,15}$). In subsequent analyses of total fatty acids recovered from pYCer1- or pYCer3-transformed cells, $16{:}2^{\Delta 6,9}$, $18{:}2^{\Delta 6,9}$, $18{:}3^{\Delta 6,9,12}$ and $18{:}4^{\Delta 6,9,12,15}$ were detected, depending on the fatty-acid precursors offered (Table 1). Yeast with pYCer1 also produced the Δ^6-acetylenic fatty acids $18{:}2A^{\Delta 6a,9,12}$ and $18{:}3A^{\Delta 6a,9,12,15}$ (see below), whereas control cells harbouring pYES2 produced none of these fatty acids. These results confirmed, that CpDes6 encodes a Δ^6-desaturase and CpAcet6 an enzyme able to function as both a Δ^6-acetylenase and a Δ^6-desaturase.

Substrate specificity

To elucidate the substrate specificity of CpDes6 and CpAcet6, transgenic yeast cultures were fed with different fatty-acid substrates. GC analysis of FAME recovered from whole cells expressing pYCer3 (CpDes6) showed, that linoleic acid (18:2) served as the best substrate for Δ^6-desaturation to γ-linolenic acid (γ-18:3) (Fig. 2). Cells harbouring pYCER1 were also able to convert incorporated 18:2 into γ-18:3, but in these cultures the newly introduced Δ^6-double bond was further converted into a triple bond, resulting in the production of 9,12-(*Z*,*Z*)-octadecadien-6-ynoic acid (18:2A). When cells with CpAcet6 were fed with γ-18:3, they also desaturated the preexisting Δ^6-double bond to form a triple bond resulting in the production of 18:2A (Fig. 2). Furthermore, the yeast cells with CpAcet6 converted stearidonic acid (18:4ω3) into 9,12,15-(*Z*,*Z*,*Z*)-octadecatrien-6-ynoic acid (18:3A), which represents the main acetylenic fatty acid found in the moss *C. pupureus* [9].

Both CpDes6 and CpAcet6 were able to desaturate α-linolenic acid (α-18:3) to stearidonic acid (18:4ω3) (Table 1). When cells were not supplemented with an exogenous fatty acid, the Δ^9-monoenoic fatty acids present in the yeast served as substrates for Δ^6-desaturation. Palmitoleic acid ($16{:}1^{\Delta 9}$) was a better substrate than oleic acid ($18{:}1^{\Delta 9}$) (Table 1). The fatty acids $16{:}3^{\Delta 7,10,13}$, $20{:}2^{\Delta 8,11}$, $20{:}3^{\Delta 8,11,14}$ and $20{:}4^{\Delta 5,8,11,14}$ did not serve as substrates for Δ^6-desaturation or Δ^6-acetylenation (data not shown). Petroselinic acid ($18{:}1^{\Delta 6}$), which has no Δ^9-double bond, was not a substrate for Δ^6-acetylenation (data not shown). From these results we concluded, that CpDes6 and CpAcet6 were able to introduce a Δ^6-double bond into Δ^9-unsaturated fatty-acid substrates, thus extending the characteristic divinylmethane pattern one step further towards the carboxyl end of the acyl chain. The CpDes6 has a strong preference for linoleic acid ($18{:}2^{\Delta 9,12}$), whereas CpAcet6 preferred γ-linolenic acid ($18{:}3^{\Delta 6,9,12}$). Palmitoleic acid ($16{:}1^{\Delta 9}$) was a better substrate for the Δ^6-acetylenase than for the Δ^6-desaturase. Therefore, the Δ^6-acetylenase seemed to be less chain length specific than the Δ^6-desaturase. A comparison (Table 1) suggests, that in yeast CpAcet6 is a better Δ^6-desaturase with a broader substrate specificity than CpDes6.

GC-MS analysis of acetylenic fatty acids

Feeding experiments with γ-18:3 to yeasts expressing either CpDes6 or CPAcet6 followed by GC-MS analysis of fatty acids were used to confirm the presence of Δ^6-olefinic and Δ^6-acetylenic bonds in the fatty acids produced by the transgenic yeasts. The methyl ester of 18:3A from the transgenic yeast expressing CpAcet6 was identical with respect to retention time (18.25 min), EI-, and CI-MS fragmentation pattern when compared with18:3A isolated from the moss *Ceratodon purpureus* and *Dicranum scoparium,* which in addition was used for NMR analysis (see below). Furthermore, the EI-MS spectrum of the 18:3A methyl ester was found to be identical with that of 9,12,15-octadecatrien-6-ynoic acid described previously [29].

These data strongly suggested the formation of 18:3A, but the position of the double and triple bonds could not be allocated unequivocally by the EI-MS analysis of 18:3A and 18:2A as their methyl ester derivatives. Therefore, FAME were converted into their DMOX-derivatives. They are considered to be the most suitable derivatives for the localization of unsaturated bonds in fatty acids and are separated by GC with similar resolution as the methyl esters [20,30]. Chemical ionization (CI)-MS analysis of the putative 18:3A (eluting at 15.85 min as compared to 14.31 min of γ-18:3) gave a pseudomolecular ion ($[M + H]^+ = 330$), which is 2 mass units (mu) lower as compared to the substrate γ-18:3 ($[M + H]^+ = 332$). This decrease in molecular mass of the new product can be ascribed either to the introduction of an additional double bond into γ-18:3 or to the desaturation of an olefinic to an acetylenic bond. It should be recalled that CPDes6 produces only γ-18:3 and stearidonic acid 18:4, whereas expression of CPAcet6 in addition leads to the formation of 18:2A and 18:3A. The EI-mass spectra of the two polyolefinic acids, γ-18:3 (Fig. 3A) and stearidonic acid (Fig. 3B) displayed molecular ions as $[M]^+$ with $m/z = 331$ (γ-18:3) and 329 (18:4), respectively, whereas the derivatives of the two corresponding acetylenic fatty acids produced pseudomolecular ions due to the loss of one hydride $[M\text{-}H]^+$ with $m/z = 328$ for 18:2A (Fig. 3C) and $m/z = 326$ for 18:3A (Fig. 3D). In addition, the series of characteristic fragment ions in the high m/z region confirms the existence of double bonds at positions 9,12 (and 15) in all four compounds (Fig. 3) including the two acetylenic fatty acids recovered from transgenic yeasts (Fig. 3C,D). On the other hand, even DMOX derivatives do not allow the unambiguous localization of a triple bond located between C2-C8. Diagnostic fragment ions of the expected size are not formed (compare Fig. 3C,D with Fig. 3A,B), and structural assignment by GC-MS has to be based on the spectral identity of the unknown and a reference compound. Therefore, we isolated the putative 18:3A in mg quantities [31] from two different mosses for independent confirmation of structural details by NMR spectroscopy and as source for obtaining the required reference mass spectrum of its DMOX derivative.

NMR spectroscopy

The ^{1}H-NMR spectra of 9,12,15-(*Z*,*Z*,*Z*)-octadecatrien-6-ynoic acid (18:3A) methylester isolated from *C. pupureus* and *D. scoparium* were found to be identical (details in Materials

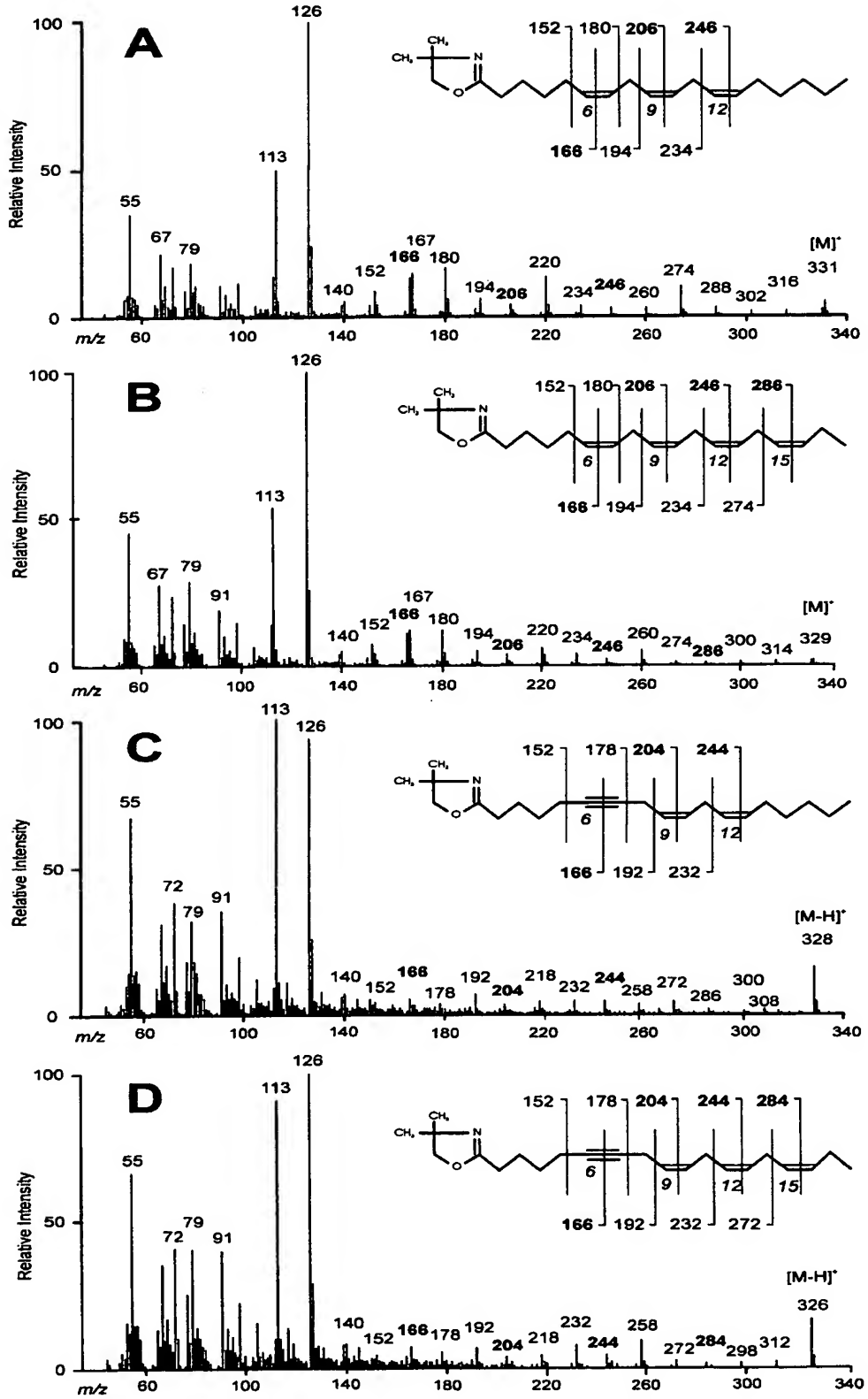

Fig. 3. Mass spectra of C18-polyenoic fatty acids with a Δ^6 double or triple bond. GC-MS (in the EI-mode) was used to obtain the spectra of DMOX derivatives from *cis*-6,9,12-octadecatrienoic acid [γ-18:3, (A)], *cis*-6,9,12,15-octadecatetraenoic acid [18:4, (B)], *cis*-9,12-octadecadien-6-ynoic (18:2A, C) and *cis*-9,12,15-octadecatrien-6-ynoic acid [18:3A, (D)]. Fatty-acid products (A, B) were isolated from transgenic yeast cells expressing either pYCer1 (Δ^6-acetylenase from *C. pupureus*) or pYCer3 (Δ^6-desaturase from *C. pupureus*) supplemented with linoleic acid (A) or γ-18:3 (B). Acetylenic acids (C, D) were isolated from cells expressing pYCer1 supplemented with either linoleic acid or γ-18:3 (C) and 18:4 (D), respectively. A gap of 12 mass units between progressively larger fragments is diagnostic for the presence of a double bond in the region between C8 and C15 (bold face), whereas desaturation and acetylenation at the Δ^6 position is indicated by the ions m/z = 152, 166, 180 and m/z = 152, 166, 178, respectively. Due to the high background between m/z 150 – m/z 190 in the spectra of the acetylenic acids, it is diffficult to recognize diagnostic fragments and, therefore, structural confirmation had to be based on comparison with spectra from reference compounds analysed by NMR (see text).

and methods). With the exception of the olefinic protons (H-9,10,12,13,15,16) all protons in 18:3A appeared as well resolved signals which allowed the complete assignment of all resonances. The presence of a triple bond between C-6 and C-7 could be deduced by a ^{1}H,^{1}H-COSY experiment showing cross peaks between H-5 (δ 2.175 p.p.m.) and H-8 (δ 2.932 p.p.m.) which originated from a diagnostic long range coupling known to be present between methylene protons separated by an acetylene group [29]. This interpretation was further corroborated by 1D ^{13}C-NMR where characteristic signals for triple bonded carbons were assigned to C-6 (δ 79.5) and C-7 (δ 78.6) which were lacking in the DEPT spectrum as expected for an acetylene group. The other signals could be assigned with the help of ^{1}H,^{13}C-HMQC and ^{1}H,^{13}C-HMBC- experiments, the latter showing unambiguously connectivities over three carbon atoms, thus further allowing assignment especially of C-6 and C-7 as well as other ^{1}H and ^{13}C NMR signals, including olefinic resonances which could not be completely achieved in previous communications [29]. Part of the 18:3A methyl ester isolated from the mosses was converted to the DMOX derivative, which gave an EI-mass spectrum identical with that shown in Fig. 3D. In conclusion, our EI-MS and NMR analysis clearly showed that both (CPDes6 and CPAcet6) can introduce a *cis*-Δ6 double bond into a suitable precursor, and CPAcet6 can further desaturate the *cis*-Δ^6 double bond to form a triple bond at this position.

DISCUSSION

Structural properties

The cDNA sequences CPAcet6 and CPDes6 encoding a novel Δ^6-acetylenase and a Δ^6-desaturase from *C. purpureus* were cloned from a cDNA library using a PCR-based approach. The deduced Δ^6-acetylenase protein shared 57% identity with the Δ^6-desaturase from *C. purpureus*, which is about the same value for the Δ^{12}-acetylenase from *C. alpina* to the Δ^{12}-desaturase from *C. palaestina* [8]. On the other hand only four amino-acid substitutions are enough to convert a Δ^{12}-desaturase into a Δ^{12}-hydroxylase [6]. These data suggest, that a similarly sized change in amino-acid composition is necessary for a switch from desaturation to acetylenation independent from regioselectivity (Δ^6 or Δ^{12}), but that it involves a more extensive change in sequence at different positions than a switch to hydroxylation. The moss Δ^6-desaturases from *C. pupureus* and *P. patens* [19] share 67% identity, but only less than 33% identity to Δ^6-desaturases from other organisms [21,26], whereas a high value (i.e. more than 64% identity for microsomal Δ^{12}-desaturases) is usually found for all desaturases sharing the same regioselectivity (Δ^9, Δ^{12} or Δ^{15}) and the same subcellular compartment, even among phylogenetically diverse species. In contrast, Δ^6-desaturases show a similarly high conservation only among species belonging to the same phylum.

The same arrangement of functional domains (a N-terminal extension, followed by a cytochrome b_5 and a C-terminal desaturase domain) present in the Δ^6-desaturase from *P. patens* [19] is verified in the Δ^6-acetylenase and in the Δ^6-desaturase from *C. pupureus,* which represents a unique feature of these moss desaturases, so far. Therefore, the Δ^6-acetylenase can be assigned as a new member of the growing family of cytochrome b_5 fusion proteins [13]. The essential role for enzymatic activity of the cytochrome b_5-domain in these fusion desaturases has been demonstrated for the yeast Δ^9-acyl-CoA desaturase [32] and for the Δ^6-acyl lipid desaturase from borage [33]. Furthermore, the presence of a cytochrome b_5-related domain suggests a microsomal rather than a plastidial localization of these enzymes, because plastidial desaturases use ferredoxin as electron donor [34]. Beside this, CPDes6 and CPAcet6 contain a N-terminal extension also found in the Δ^6-desaturase from *P. patens* (about 52–95 amino acids) [19], which is absent in other presently known desaturases. The function of this extension is unclear, as it shows no significant homology to any known protein or targeting sequence. The desaturase domain downstream of the cytochrome b_5 of both proteins shows the three histidine boxes conserved in all desaturases [2]. Furthermore, in the Δ^6-acetylenase and Δ^6-desaturase from *C. pupureus* the first histidine of the third box appearing in all Δ^9-, Δ^{12}- and Δ^{15}-desaturases is substituted by a glutamine residue (QXXHH). This substitution seems to be a common feature of all N-terminal cytochrome b_5 fusion desaturases with a Δ^5-, Δ^6- or Δ^8-regioselectivity, known so far. As for these fusion desaturases, the Δ^6-acetylenase can be assigned as 'front-end' desaturase, which recognizes a pre-existing double bond at the Δ^9 position (x) and introduces a new double and triple bond, respectively, extending the divinylmethane pattern ($x - 3 = \Delta^6$ position) towards the carboxyl end of the fatty acyl chain.

Functional analysis in *S. cerevisiae*

The expression of CPDes6 and CPAcet6 in *S. cerevisiae* with or without supplementation of different fatty-acid substrates resulted in newly formed desaturation products neither present in control cells transformed with the empty vector nor in wild-type cells. The structure including the double and the triple bond positions, respectively, of these newly formed fatty-acid products were confirmed by GC-MS of their DMOX-derivatives. Based on the presented data, we conclude that CPDes6 codes for a Δ^6-desaturase and that CPAcet6 codes for a Δ^6-acetylenase, both of which are able to desaturate Δ^9-unsaturated C16- and C18-fatty acids, resulting in the production of $18{:}3^{\Delta6,9,12}$, $18{:}4^{\Delta6,9,12,15}$, $16{:}2^{\Delta6,9}$ and minor amounts of $18{:}2^{\Delta6,9}$. A similar substrate specificity for $\Delta^{9,12(15)}$-polyenoic and Δ^9-monoenoic acids has also been observed for the Δ^6-desaturases from *P. patens* [19], *M. alpina* [21] and *C. elegans* [23]. Apart from the same broad substrate specificity of a Δ^6-desaturase, CPAcet6 can further generate a triple bond by desaturating $18{:}3^{\Delta6,9,12}$ to $18{:}2A^{\Delta6a,9,12}$ and $18{:}4^{\Delta6,9,12,15}$ to $18{:}3A^{\Delta6a,9,12,15}$. Based on these data, we assume that CPAcet6 codes for a bifunctional Δ^6-acetylenase/desaturase, which can use both $\Delta^{9(12)}$-unsaturated fatty acids for the introduction of a double bond and, independent from this, $\Delta^{6,9,12(15)}$-unsaturated substrates for the formation of a triple bond. At least in yeast, the occurrence of concomitant Δ^6-desaturation might be due to a limited supply of the preferred $\Delta^{6,9,12(15)}$-unsaturated fatty-acid substrates for acetylenation. On the other hand, there are more examples for bifunctional enzymes. When expressed in yeast, the *C. alpina* acetylenase [8] and the *L. fendleri* hydroxylase [5] showed Δ^{12}-desaturase activity, the *A. thaliana* Δ^{12}-oleate desaturase [6] lost its chain length specificity by using palmitoleic acid and the Δ^8-sphingolipid desaturases from *Brassica napus* and *A. thaliana* [35] lacked stereoselectivity by introducing a *cis*- and *trans*-double bond, respectively. In yeast expressing CPAcet6 18:2 was desaturated two times at the same positions to yield 18:2A. Furthermore, γ-18:3 was a better substrate for acetylenation than 18:4, whereas α-18:3 only served as a substrate for Δ^6-desaturation but not for Δ^6-acetylenation. These data are in agreement with the pathway suggested for

18:3A synthesis in the moss *C. pupureus*: linoleic acid (18:2) is desaturated twice in the Δ^6 position, yielding 18:2A, which undergoes a Δ^{15}-desaturation to yield 18:3A [9]. At present, very little is known about the catabolism and function of Δ^6-acetylenic fatty acids in plants. Recently, an acetylene hydratase was purified from *Pelobacter acetylenicus* catalyzing the degradation of acetylene to acetaldehyde [36].

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Identification of a novel Δ6-acyl-group desaturase by targeted gene disruption in *Physcomitrella patens*

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Summary

The moss *Physcomitrella patens* contains high levels of arachidonic acid. For its synthesis from linoleic acid by desaturation and elongation, novel Δ5- and Δ6- desaturases are required. To isolate one of these, PCR-based cloning was used, and resulted in the isolation of a full-length cDNA coding for a putatively new desaturase. The deduced amino acid sequence has three domains: a N-terminal segment of about 100 amino acids, with no similarity to any sequence in the data banks, followed by a cytochrome b_5-related region and a C-terminal sequence with low similarity (27% identity) to acyl-lipid desaturases. To elucidate the function of this protein, we disrupted its gene by transforming *P. patens* with the corresponding linear genomic sequence, into which a positive selection marker had been inserted. The molecular analysis of five transformed lines showed that the selection cartridge had been inserted into the corresponding genomic locus of all five lines. The gene disruption resulted in a dramatic alteration of the fatty acid pattern in the knockout plants. The large increase in linoleic acid and the concomitant disappearance of γ-linolenic and arachidonic acid in all knockout lines suggested that the new cDNA coded for a Δ6-desaturase. This was confirmed by expression of the cDNA in yeast and analysis of the resultant fatty acids by GC–MS. Only the transformed yeast cells were able to introduce a further double bond into the Δ6-position of unsaturated fatty acids. To our knowledge, this is the first report of a successful gene disruption in a multicellular plant resulting in a specific biochemical phenotype.

Introduction

Compared to higher plants, many members of moss, algae and fern families produce a wider variety of polyunsaturated fatty acids (PUFA; Dembitsky, 1993; Jamieson and Reid, 1975; Zhukova and Aizdaicher, 1995), and PUFA such as arachidonic acid (AA) and eicosapentaenoic acid (EPA) are produced only by lower plants. The function of these long-chain PUFA in the membranes of lower plants is still unclear, whereas in humans, they play a key role in eicosanoid metabolism (Samuelsson, 1983).

The biosynthesis of AA and EPA generally starts with linoleic acid (18:2), which is channelled into a widely branching network of desaturation and elongation steps (Arao and Yamada, 1994; Cohen *et al.*, 1995; Shiran *et al.*, 1996). Key enzymes in this network are Δ5- and Δ6-desaturases, which introduce the new double bond between the first double bond and the carboxyl terminus of the fatty acid, known as carboxyl-directed desaturation. This mode differs from the methyl-directed desaturation, which works towards the methyl end of the unsaturated fatty acid. Desaturases of both types belong to the membrane-bound desaturases, which operate in microsomes or in plastids (Heinz, 1993). All desaturases, including acyl-ACP, (Ohlrogge *et al.*, 1993), acyl-CoA (Enoch *et al.*, 1976) and acyl-lipid desaturases, are believed to catalyse an O_2-dependent reaction, in which either cytochrome b_5 serves as electron donor for the microsomal or ferredoxin for the plastidial desaturases (Kearns *et al.*, 1991; Schmidt and Heinz, 1990; Smith *et al.*, 1990).

In the last few years, extensive sequence information from various desaturases in the methyl-directed group has been accumulated, but only a few from the carboxyl-directed group (Reddy *et al.*, 1993; Sayanova *et al.*, 1997) have been cloned so far. A good source to clone new desaturases is the moss *Physcomitrella patens*. Lipids of *P. patens* contain high proportions of AA (up to 30% of total fatty acids) indicating strong expression of Δ5- and Δ6-desaturases (Grimsley *et al.*, 1981). This moss can be propagated vegetatively in the haploid state (Ashton and Cove, 1977), which simplifies the phenotypic analysis after mutation or transformation (Schaefer *et al.*, 1991). Genes of this organism can be specifically inactivated by gene targeting, as shown by Schaefer and Zryd (1997), who demonstrated that integration of homologous DNA into the genome of *P. patens* takes place by homologous recombination with a relative efficiency of more than 90% among transgenic plants.

In the present communication, we describe the isolation of a new cDNA and its corresponding genomic sequence from *P. patens*, using a PCR-based screening. The encoded protein shared less than 27% sequence identity with known desaturases and represents a fusion between a C-terminal

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desaturase with a cytochrome b_5-related part and a N-terminal extension. Its function and importance for the biosynthesis of AA (20:4) was identified by disrupting the corresponding gene in *P. patens*. The biochemical phenotype of the null mutant and its subsequent complementation by feeding γ-linolenic acid ($18:3^{\Delta 6,9,12}$) demonstrated that the disrupted gene codes for a Δ6-desaturase, which plays a key role in the synthesis of 20:4.

Results

PCR-based cloning

For PCR experiments, different sets of degenerate primers, deduced from the three conserved histidine boxes of acyl-lipid desaturases, were synthesized (Avelange-Macherel *et al.*, 1995; Shanklin *et al.*, 1994). The template used was single-stranded cDNA from *P. patens*, which was reverse-transcribed from mRNA of 12-day-old protonema cultures. Bands of the expected length were cloned and sequenced. Data bank searches and alignments with these new sequences indicated similarities to acyl-lipid desaturases for seven cDNA fragments. Six of them were classified as putative members of the well-known Δ12- and Δ15-desaturases based on high identities of over 60%. In contrast to this, one sequence of 550 bp showed less than 27% identity to known desaturases. Since *Physcomitrella* was expected to express Δ5- and Δ6-desaturases, it was postulated that this sequence might be derived from one of those desaturases.

Isolation of a full-length cDNA

To isolate a full-length cDNA clone, the 520 bp PCR fragment was DIG-labelled, and used to screen a cDNA library of 12-day-old protonemta. Of 3.0×10^5 plaques screened, 19 positives were isolated. The restriction analysis of their inserts showed a similar pattern in all cases. The partial sequence analysis from six inserts revealed that they were identical to each other within their overlapping regions and also to the original 520 bp PCR fragment. The longest insert, designated *PPDES6* cDNA, was sequenced on both strands. It had a length of 2012 bp excluding its poly(A) tail. An open reading frame stretched from position 319–1894, and several stop codons in the corresponding 5′ untranslated region indicated its full length (Figure 1). The protein PPDES6 translated from the *PPDES6* cDNA contained 525 amino acid residues with a calculated molecular weight of 59.3 kDa. This is 7–20 kDa larger than all acyl-lipid desaturases known from higher plants and cyanobacteria. Data bank searches indicated similarity to cytochrome b_5 sequences from residues 105–176 and to desaturases from residue 207 towards the C-terminus.

The desaturase domain showed the highest similarity to the cytochrome b_5-containing fusion protein of *Helianthus annuus* (Sperling *et al.*, 1995), a putative fusion protein from *Caenorhabditis elegans* encoded by cosmid T13F2 (Z81122) and the Δ6-desaturases of *Spirulina platensis* (X87094), *Borago officinalis* (Sayanova *et al.*, 1997) as well as *Synechocystis* sp. PCC 6803 (Reddy *et al.*, 1993). The identity values of PPDES6 to these proteins were low and ranged from 21% to 27% for the sequence between the first and third histidine boxes and from 12% to 23% over the entire length. The sequence motive QIEHH of the third histidine box started with a glutamine instead of a histidine, which has also been found in Δ6-desaturases and the cytochrome b_5 fusion protein of *H. annuus*, but not in other membrane-bound desaturases. The hydrophobicity plot (Kyte and Doolittle, 1982) after residue 200 showed the typical profile of membrane-bound desaturases (data not shown). The cytochrome b_5-related domain contained the eight invariant residues typical for the cytochrome b_5 superfamily (Lederer, 1994).

The N-terminal extension of about 100 residues did not share significant similarity to any sequence in the data banks, and computer analysis did not detect any motives for protein targeting or modification either for the extension or for the whole protein.

Structure of the gene

To knock out the *PPDES6* gene, its genomic sequence was amplified by PCR with specific primers C and D. Primer C was deduced from the 5′ end and D from the middle of the 3′ untranslated region of the *PPDES6* cDNA. PCR with these primers and genomic DNA of *P. patens* as template amplified a fragment that was 1578 bp longer than the distance between the binding sites of the primers on the cDNA. The genomic PCR fragment, denoted *PPDES6*, was cloned and sequenced on both strands (Figure 2). Apart from six putative introns (i1-i6) it was 100% identical with the cDNA, confirming its identity as the genomic locus of the *PPDES6* cDNA. The 5′ splicing border of five introns was GT and the 3′ border of all six was AG. Only the fourth intron i4 contained the unusual 5′ splicing border GC, which has been found in genes of several plant species (Xue and Rask, 1995). The reliability of this intron sequence was confirmed by sequencing two other PCR-amplified clones over this region. The intron i4 was located between two triplets coding for residues 176 and 177. After residue 176 the detected similarity to cytochrome b_5 sequences was terminated.

Gene targeting

For the disruption experiments, the first histidine box of the genomic clone was replaced by the *npt* II gene as a positive selection marker. The subsequent double digestion

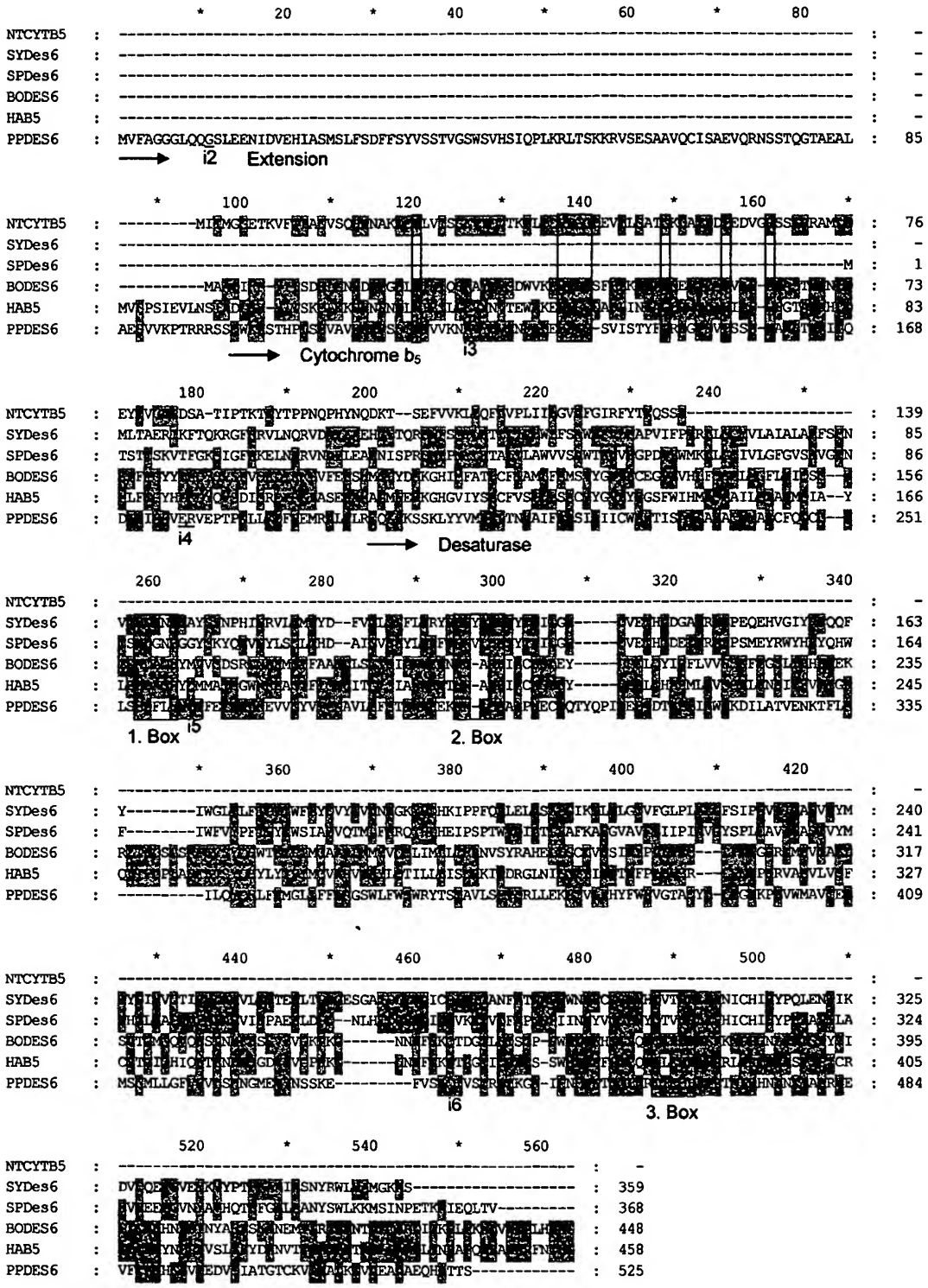

Figure 1. Amino acid sequences of PPDES6 and closely related proteins.
For alignment the CLUSTAL X program was used (gap opening 10, gap extension 0.05). Conserved and invariant residues are grey. The approximate beginning of the three domains from PPDES6 are marked by arrows and their putative function. The eight invariant residues characteristic for the cytochrome b_5 superfamily and the three histidine boxes of the desaturase domains are framed. The underlined residues indicate the positions of introns i1–i6 in the genomic sequence *PPDES6*. SYDes6, SPDes6 and BODES6 refer to the Δ6-desaturases of *Synechocystis* (U79010), *Spirulina* (X87094) and *Borago* (U79010). NTCYTB5 and HAB5 refer to the cytochrome b_5 of *Nicotiana* (X71441) and the b_5 fusion protein of *Helianthus* (X87143), respectively.

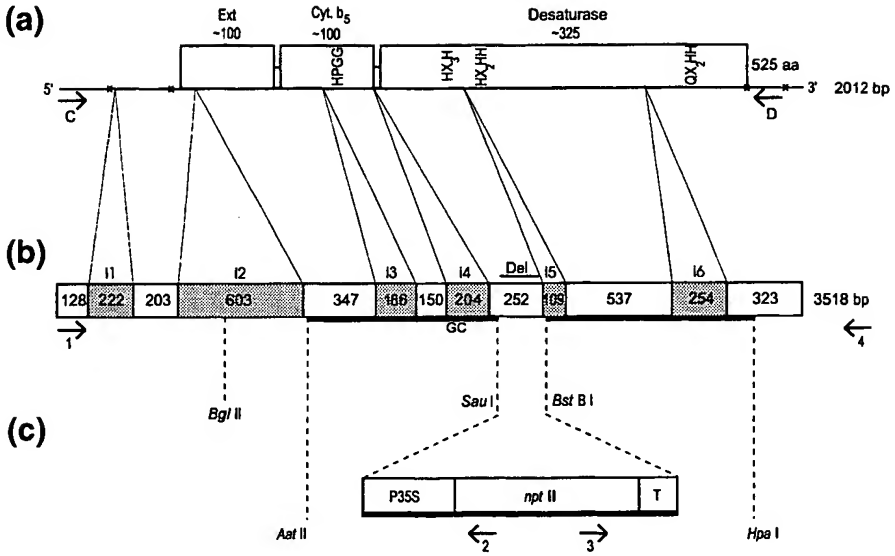

Figure 2. Structure of the desaturase cDNA (a), the desaturase gene (b) and the fragment used for gene disruption (c).
(a) *PPDES6* cDNA (2012 bp) and schematic representation of the protein PPDES6 (525 aa) with three domains: an extension (Ext) of unknown function, a cytochrome b_5- and a desaturase-like region with typical amino acid blocks. Stop codons are symbolized by stars.
(b) Corresponding genomic sequence *PPDES6* amplified with primer C and D (shown in a). The positions and lengths of the six introns (i1–i6, grey) are marked. The GC splice junction is shown for i4.
(c) Replacement fragment for gene disruption: the first histidine box in the genomic fragment was substituted after a *Sau*I/*Bst*BI double digestion by the *npt* II cartridge, which contained the *npt* II coding region between the CaMV 35S promoter (P35S) and terminator (T). The transformation was carried out after linearization with *Aat*II and *Hpa*I resulting in a linear fragment (underlined in bold) with the *npt* II cartridge inserted into the sequence of the desaturase. The numbered arrows below the blocks indicate the binding sites of primers used for subsequent PCR analyses. The localization of the Del probe used for Southern blotting (see Figure 3) is marked with a line above the block.

with *Sau*I/*Bst*BI yielded a linear fragment with the *npt* II gene in its centre and the desaturase arms at both ends (Figure 2). This linear fragment was used to transform *P. patens* protoplasts by the PEG method (Schaefer *et al.*, 1991). Seven transformation experiments with 3.0×10^5 protoplasts in each experiment resulted in the isolation of 56 independent and stably transformed lines. Five randomly selected transgenic lines (K1–K5) were used for detailed analysis regarding the molecular biology of gene disruption as well as its consequences for fatty acid biosynthesis.

Molecular analysis of the transgenic lines

The specific integration of the transformed DNA into the *PPDES6* gene was analysed by PCR using genomic DNA from five transformed lines (K1–K5) and the wild type. The locations of the different primers are presented in Figure 2. It is important to point out that the 3′ end of primer 4 binds 40 bp downstream of the cloned genomic sequence to exclude PCR signals resulting from contamination by the DNA used for transformation. Its sequence was derived from the 3′ end of an incomplete cDNA clone, which showed the same sequence in the overlapping region with cDNA *PPDES6*, but contained a longer 3′ end.

PCR with the primer pair 1/2 amplified fragments of 2.7 kbp, and with the primer pair 3/4 bands of 1.6 kbp, from all five transformants, whereas experiments with the wild type gave negative results. The length of the bands agreed with a substitution of the first histidine box of the *PPDES6* gene by the *npt* II cassette. Both PCR fragments from two transformants (K2 and K3) were cloned and partially sequenced. The sequenced segments were identical with the corresponding regions of the transformed gene disruption construct. Most important, the fragments from primer pair 3/4 contained the downstream genomic element of 40 bp, which was absent in the transformed DNA. They lacked the first histidine box, and the transition regions of the *npt* II cassette to the *PPDES6* gene, as well as the regions containing the restriction sites *Aat* II and *Hpa* I, were identical in their sequence with the disruption construct.

To provide evidence for a deletion of the first histidine box in the *PPDES6* gene of the transgenic lines, the genomic DNA of the transformed lines and the wild type was digested with *Bgl*II, blotted and hybridized with the DIG-labelled deletion probe Del. This probe represents the *Sau*I/*Bst*BI fragment encoding the first histidine box, which had been deleted from the transformed disruption construct (Figure 3). Hybridization with the deletion probe Del showed one strong signal of 4.5 kbp and two very weak signals of 5.0 and 7.0 kbp with the wild type DNA. The transformed lines K1–K4 had lost the strong 4.5 kbp signal but not the two weak signals. Line K5 corresponded to the

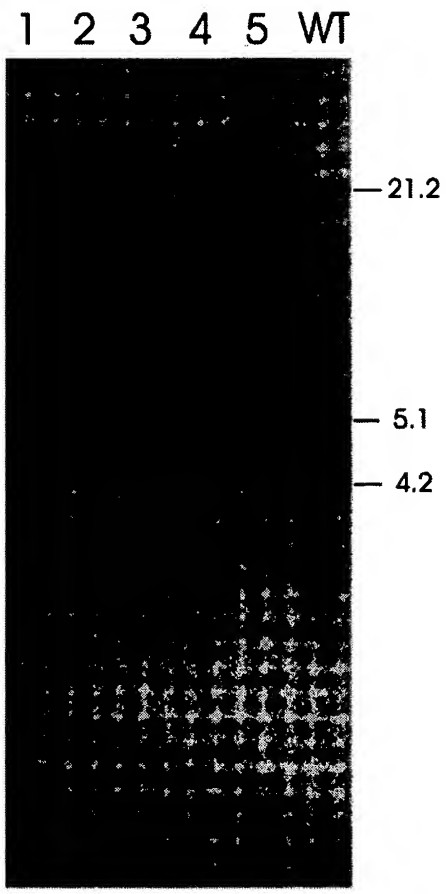

Figure 3. Verification of gene disruption by Southern blotting.
Genomic DNA (4 μg) from the wild type (WT) and five transformed lines K1–K5 (1–5) was digested with *Bgl*II and hybridized with the deletion probe (Del). The location of the probe is described in Figure 2. Molecular weights in kbp are indicated on the right.

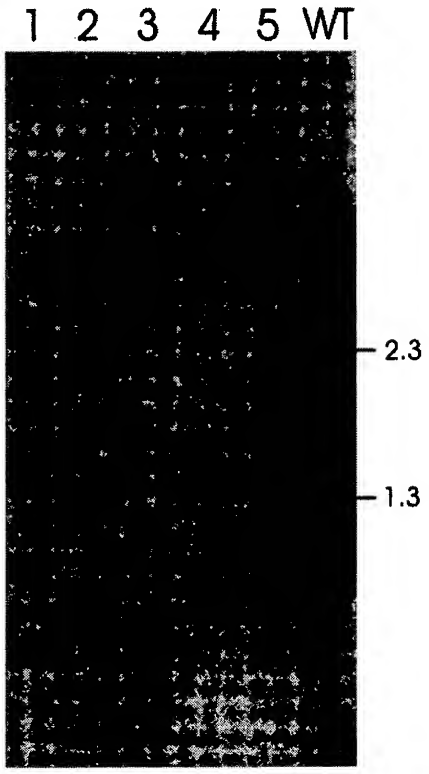

Figure 4. Northern blot analysis of *PPDES6* expression.
Total RNA (20 μg) from 14-day-old *P. patens* protonemata was probed with an RNA probe transcribed from the last 600 bp of the *PPDES6* cDNA. Five transgenic lines K1–K5 (1–5) and the wild type (WT) were analysed. Molecular weights in kb are indicated on the right.

wild type situation but contained an additional band of more than 21 kbp.

To compare the expression of *PPDES6* in the five transgenic lines with the wild type, we blotted total RNA of 14-day-old protonemata and hybridized it with a DIG-labelled RNA probe against the 3′ end of the *PPDES6* cDNA (Figure 4). The wild type showed a strong signal of 2.0–2.2 kb, whereas the five transgenic lines had lost this transcript. Hybridization with a *npt* II-specific probe (blot not shown) detected a strong signal of 1.0–1.3 kb in all transgenic lines but not in the wild type.

Functional analysis of PPDES6 in P. patens

For the functional identification of the desaturase, we analysed the total fatty acids of the wild type and the five knockout lines. The fatty acid analyses presented in Figure 5 are confined to the wild type and to line K2, but the other four lines tested gave essentially the same results. Pathways [1] and [2] below show the sequences proposed for the biosynthesis of AA (20:4) and EPA (20:5) in *P. patens*, and they are supported by our results (fatty acids are indicated as $m{:}n^{\Delta a,b,c,\cdots}$; m refers to the number of carbon atoms, n to the double bonds and $^{\Delta a,b,c,\cdots}$ to the position of the double bonds; desaturation and elongation steps are indicated by Δx and EL).

$$18{:}2^{\Delta 9,12} \xrightarrow{\Delta 6} 18{:}3^{\Delta 6,9,12} \xrightarrow{EL} 20{:}3^{\Delta 8,11,14} \xrightarrow{\Delta 5} 20{:}4^{\Delta 5,8,11,14} \quad [1]$$

$$\downarrow \Delta 15$$

$$18{:}2^{\Delta 9,12,15} \xrightarrow{\Delta 6} 18{:}4^{\Delta 6,9,12,15} \xrightarrow{EL} 20{:}4^{\Delta 8,11,14,17} \xrightarrow{\Delta 5} 20{:}5^{\Delta 5,8,11,14,17} \quad [2]$$

Compared with the wild type, all transgenic lines showed a strong decrease in those unsaturated fatty acids, the formation of which involves a Δ6-desaturation step (Figure 5): $18{:}3^{\Delta 6,9,12}$, $18{:}4^{\Delta 6,9,12,15}$, $20{:}3^{\Delta 8,11,14}$, $20{:}5^{\Delta 5,8,11,14,17}$ and most clearly $20{:}4^{\Delta 5,8,11,14}$. On the other hand, the possible substrates for a Δ6-desaturase, $18{:}2^{\Delta 9,12}$ and $18{:}3^{\Delta 9,12,15}$, increased. Therefore, it is most likely that the reactions from $18{:}2^{\Delta 9,12}$ to $18{:}3^{\Delta 6,9,12}$ as well as from $18{:}3^{\Delta 9,12,15}$ to $18{:}4^{\Delta 6,9,12,15}$ were blocked, both of which are catalysed by a Δ6-desaturase (compare pathways [1] and [2]).

To provide further evidence for the function of the new

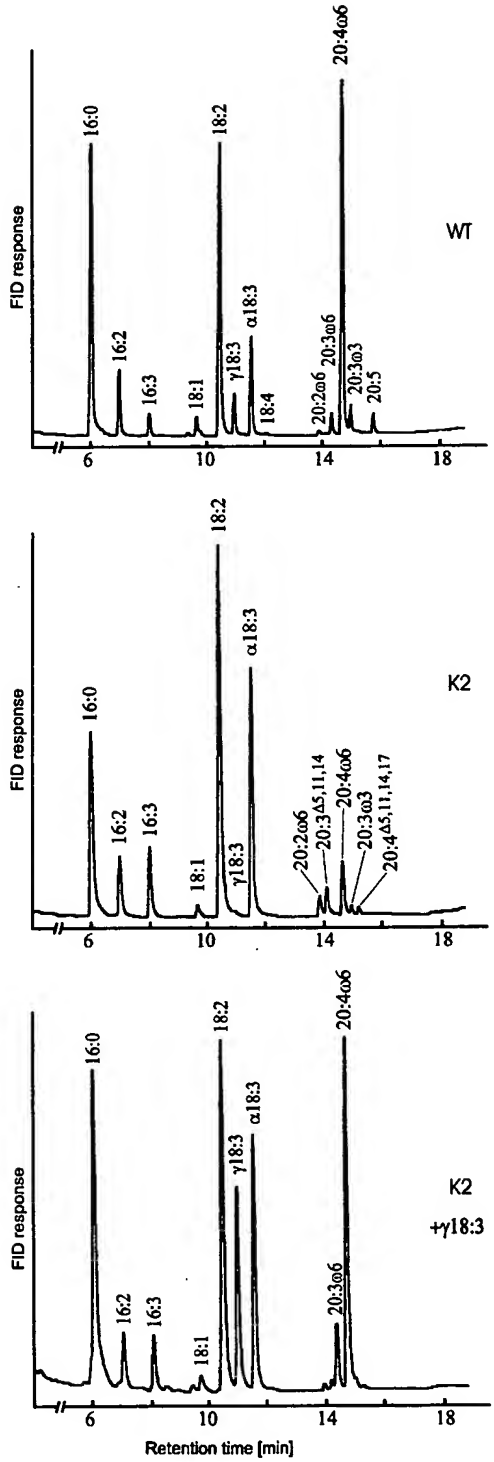

Figure 5. Fatty acid profiles of the *P. patens* wild type (WT) and the knockout line K2. The fatty acid methyl esters (FAME) of the total lipids were analysed by capillary gas-liquid chromatography. The chromatograms WT and K2 show the FAME of protonemata grown for 14 days in liquid medium. The lower chromatogram shows the FAME profile of K2 cells cultured under the same conditions but in the presence of 50 μM of γ18:3 ($18:3^{\Delta 6,9,12}$).

Δ6-desaturase, we supplemented the knockout line K2 and the wild type with $18:3^{\Delta 6,9,12}$ (γ18:3). In K2 the feeding of this fatty acid resulted in the reappearance of $20:3^{\Delta 8,11,14}$ and $20:4^{\Delta 5,8,11,14}$, whereas almost no change was observed in the wild type. This experiment indicates that the knockout line K2 is able to synthesize 20:4 from added $18:3^{\Delta 6,9,12}$, but not from $18:2^{\Delta 9,12}$, which increases in unsupplemented K2. However, the addition of $18:3^{\Delta 6,9,12}$ did not result in a complementation of the almost complete disappearance of $20:5^{\Delta 5,8,11,14,17}$ in K2.

The addition of $20:2^{\Delta 11,14}$ and $20:3^{\Delta 11,14,17}$ (data not shown) did not result in an increase of 20:4 and 20:5 in the wild type or in K2. Another interesting effect of the knockout was the completely different proportion of C20-fatty acids in K2 (7%) compared to the wild type (30%).

Functional expression of PPDES6 in Saccharomyces cerevisiae

To exclude the possibility that the loss of a Δ6-desaturase in the knockout lines is a consequence of a regulatory difference between the *Physcomitrella* wild type and knockout lines, *PPDES6* was functionally expressed in *Saccharomyces cerevisiae*. Plasmid pYESΔ6 containing the open reading frame of the *PPDES6* cDNA was transformed into the *S. cerevisiae* strain INVSC1. One clone transformed with pYESΔ6 and another with the empty vector pYES2 as control were grown for four to five generations after induction with 2% galactose in minimal medium. Since *S. cerevisiae* does not contain the dienoic fatty acid substrates required for a Δ6-desaturase, the expression was performed with supplementation of $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$, respectively. In subsequent analyses of total fatty acids, the following Δ6-desaturated products were detected in the strain expressing PPDES6: $16:2^{\Delta 6,9}$, $18:2^{\Delta 6,9}$, $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ (Table 1). In the control cells, none of these fatty acids were detected. The production of these fatty acids with an additional Δ6-double bond confirmed that cDNA *PPDES6* encodes a Δ6-fatty acid desaturase.

Discussion

Structural properties

The cDNA and the genomic sequence *PPDES6* encoding a novel Δ6-desaturase from *P. patens* were cloned using a PCR-based approach. The deduced protein shared less than 27% identity with the recently cloned Δ6-desaturase from *B. officinalis* and with the Δ6-desaturases from cyanobacteria (Reddy *et al.*, 1993; Sayanova *et al.*, 1997). This is a surprisingly low value, as until now all desaturases of the same regioselectivity and the same subcellular compartment have been more highly conserved, even between distantly related organisms. For example, six

Table 1. Expression of the Δ6-desaturase in *S. cerevisiae*. The fatty acid methyl esters of the total lipids from cells transformed with pYES2 (WT control) and pYESΔ6 (Δ6-desaturase of *P. patens*) were analysed by GLC. The cells were cultured in minimal medium supplemented with 2% galactose for 24 h at 30°C. The last two columns show data from cultures supplemented with $18{:}2^{\Delta 9,12}$ (18:2) and $18{:}3^{\Delta 9,12,15}$ (α18:3)

	% total fatty acids			
	pYES2	pYESΔ6		
Fatty acids	–	–	+ 18:2	+ α18:3
16:0	16.4	16.1	23.8	25.8
$16{:}1^{\Delta 9}$	54.0	55.5	38.1	31.4
$16{:}2^{\Delta 6,9}$	–	4.2	1.7	–
18:0	3.2	2.4	4.0	4.7
$18{:}1^{\Delta 9}$	24.9	19.7	19.1	19.2
$18{:}2^{\Delta 6,9}$	–	0.6	0.2	–
$18{:}2^{\Delta 9,12}$	–	–	8.5	–
$18{:}3^{\Delta 6,9,12}$	–	–	4.0	–
$18{:}3^{\Delta 9,12,15}$	–	–	–	11.7
$18{:}4^{\Delta 6,9,12,15}$	–	–	–	3.0

other PCR fragments from *P. patens*, isolated in this screening, coded for putative Δ12- and Δ15-desaturases and displayed more than 60% identity to the corresponding desaturases of higher plants and cyanobacteria.

The presence of the cytochrome b_5-related domain upstream of the desaturase suggests its localization in microsomes rather than in chloroplasts, because plastidial desaturases normally use ferredoxin as electron donor (Heinz, 1993). Besides this, PPDES6 contains a new N-terminal extension of about 100 amino acids, which is absent in other presently known desaturases. The function of this extension is unclear, since it shows no significant homology to any known protein, and targeting or modification signals were not detected. Interestingly, the three histidine boxes and the cytochrome b_5 domain of PPDES6 are encoded by separate exons (Figure 2), implying that they may constitute separate evolutionary units. The fourth intron containing the unusual 5′ splicing border GC is located directly after the last triplet for the cytochrome b_5 domain. This organization could allow a differential splicing between the 5′ border of the first and the 3′ border of the fourth intron, resulting in a deletion of both the cytochrome b_5 domain and the N-terminal extension from the desaturase domain of the *PPDES6* transcript.

Molecular analysis of the transgenic lines

In this study, we have described the highly efficient knockout of the *PPDES6* gene after transforming *P. patens* with a linear disruption fragment. PCR experiments proved the specific integration of the *npt* II cassette into the *PPDES6* locus in all arbitrarily chosen transgenic lines. Furthermore, Southern blot experiments confirmed the deletion of a 200 bp segment encoding the first histidine box from the genome of four transgenic lines (K1–K4). It is likely that reciprocal exchange by double cross-over led to the integration observed in these four lines. Targeting experiments from Schaefer and Zrÿd (1997) demonstrated homologous integration into a locus but not a substitution. The blots with line K5 reveal an even more complicated situation. Nevertheless, K5 does not express the Δ6-desaturase activity any more. Two additional signals of low intensity in wild type and in all transgenic lines indicated that related genomic sequences were not involved in the gene targeting events. The presence of these sequences suggests that isoforms of other Δ6-desaturases could be expressed to some extent in the knockout lines.

In the Northern blots all transgenic lines showed a dramatically reduced expression of *PPDES6* while this transcript was abundant in the wild type. Thus loss of desaturase activity, as evident from the fatty acid profiles most probably resulted from loss of transcription due to gene disruption.

Functional analysis of PPDES6 in P. patens *and* S. cerevisiae

The gene disruption of *PPDES6* resulted in a dramatic alteration of the fatty acid pattern in the transformed lines. The knockout lines showed an increase of 18:2 and α18:3 and a decrease of Δ6-desaturated fatty acids. Therefore, it is likely that *PPDES6* codes for a Δ6-desaturase, which desaturates $18{:}2^{\Delta 9,12}$ to $18{:}3^{\Delta 6,9,12}$ and $18{:}3^{\Delta 9,12,15}$ to $18{:}4^{\Delta 6,9,12,15}$. The Δ6-regioselectivity of PPDES6 was further verified by restoration of 20:4 biosynthesis upon feeding of γ18:3 (Figure 5). The synthesis of 20:4 from γ18:3 would not work if a Δ5-desaturase or the elongation system had been blocked. The Δ6-desaturation of 18:2 and α18:3 added to *S. cerevisiae* cells expressing PPDES6 confirmed these results and excluded the possibility that the loss of a Δ6-desaturase in the knockout lines was due to regulatory alterations, for example the loss of an activator for the Δ6-desaturase. On the other hand, we could not detect a Δ8-C20-desaturase in *P. patens*, since addition of $20{:}2^{\Delta 11,14}$ and $20{:}3^{\Delta 11,14,17}$ did not increase the content of 20:4 and 20:5. A Δ8-desaturase operating at the C20-level could theoretically replace the Δ6-C18-desaturase in the biosynthesis of 20:4 and 20:5. Such an enzyme has been suggested to be present in *Euglena gracilis* (Nichols and Appleby, 1969).

Based on the knockout effects and feeding experiments, we propose the two pathways [1] and [2] mentioned above for the biosynthesis of 20:4 and 20:5 in *P. patens*, which branch at 18:2. They are in agreement with the biosynthesis of 20:4 and 20:5 as suggested for *Porphyridium cruentum* (Shiran *et al.*, 1996).

It should be noted that *S. cerevisiae* cells expressing PPDES6 produced not only 18:3$^{\Delta6,9,12}$ and 18:4$^{\Delta6,9,12,15}$, but also 16:2$^{\Delta6,9}$ and 18:2$^{\Delta6,9}$, which were not detected in *P. patens*. The reason for their absence in *P. patens* may be the low content and rapid turn-over of the putative precursors, 16:1$^{\Delta9}$ and 18:1$^{\Delta9}$, in the moss, whereas they are produced in high amounts by *S. cerevisiae*. Since the Δ6-desaturase converts 16:1$^{\Delta9}$ to 16:2$^{\Delta6,9}$, but does not introduce a Δ8-double bond into 20:2$^{\Delta11,14}$ and 20:3$^{\Delta11,14,17}$ (mentioned above), the insertion of the Δ6-double bond involves measuring from the carboxy terminus (and the Δ9-double bond) rather than from the methyl end. This classifies the desaturase as a Δ6-desaturase (Heinz, 1993).

Another interesting effect is the significant decrease in C20-fatty acids in the knockout lines. The decrease from more than 30% in the wild type to less than 7% in K2 indicates that the elongation system of *P. patens* prefers or even requires Δ6-desaturated C18-fatty acids. This elongation process is either very rapid or channelled and thus prevents the accumulation of γ18:3 or 18:4 in lipids. In the other organisms, from which Δ6-desaturases have been cloned (*B. officinalis* and *Synechocystis*), elongation systems do not co-operate with this desaturase and therefore Δ6-desaturated fatty acids can accumulate. A detailed analysis of lipids and fatty acids in *P. patens* wild type and knockout plants, as well as in *S. cerevisiae* expressing the Δ6-desaturase, will be published elsewhere (T. Girke *et al.*, manuscript in preparation).

In our present study, all knockout lines still contained small amounts of fatty acids, which were synthesized by a pathway requiring Δ6-desaturase. This indicates that at least one other functional gene for a Δ6-desaturase should exist. Possible candidates may be the two faint signals observed above the targeted 4.5 kbp fragment in Southern blots of wild type and transgenic lines (Figure 3).

Apart from these biochemical changes, we did not detect any visibly altered phenotype in the knockout plants, at least in their protonema or gametophore states at 25°C. Therefore, it was not possible at this point to evaluate the physiological importance of 20:4 for the moss. The appearance of a visible phenotype may also be prevented by residual 20:4. Deletions of several desaturases in *Synechocystis* became critical only if the Δ6- and Δ12-desaturase were knocked out together, whereas a reduction in trienoic acids without affecting dienoic acids was not critical (Tasaka *et al.*, 1996).

Experimental procedures

Plant material and culture conditions

The protonemata of *Physcomitrella patens* (Hedw.) BSG were grown in liquid medium (Reski *et al.*, 1994). For feeding experiments with fatty acids, 4-day-old cultures were supplemented with ammonium salts of fatty acids (dissolved in ethanol) to a final concentration of 50 μM and further cultivated for an additional 6–8 days.

Analysis of nucleic acids

DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989) unless otherwise stated. DNA sequences were determined on both strands by the dideoxy chain termination method using Dye Primer as well as Dye Terminator sequencing kits.

PCR with degenerated primers and cDNA library screening

Poly(A)$^+$ RNA was isolated with Dynabeads (Dynal, Oslo, Norway) from total RNA of 12-day-old *P. patens* protonema cultures, and reverse-transcribed into single-stranded cDNA. This ss-cDNA was used as template in the PCR-based cloning. A 550 bp PCR fragment was amplified with the degenerate sense primer A 5′-TGGTGGAA (A/G)TGGA(C/A)ICA(T/C)AA-3′ and antisense primer B 5′-GG (A/G)AA(A/T/G/C)A(A/G)(G/A)TG(G/A)TG(C/T)TC-3′ derived from the amino acid sequence WWKW (N/T)HN and EHHLFP, respectively. The PCR reactions were carried out with Taq DNA polymerase using an amplification programme of 3 min denaturation at 94°C, followed by 30 cycles of 20 sec at 94°C, 30 sec at 45°C, 1 min at 72°C and terminated by 5 min extension at 72°C. The PCR fragments of the expected length (500–600 bp) were cloned in pUC18 and sequenced. A digoxygenin-labelled DNA probe of the PCR fragment was synthesized by PCR and used to screen a lambda ZAPII cDNA library of 12-day-old protonemata according to the manufacturer's protocols (Boehringer, Mannheim, Germany; Stratagene, La Jolla, CA). The longest insert (*PPDES6* cDNA) was sequenced on both strands using overlapping subclones. The corresponding genomic sequence *PPDES6* was isolated by PCR with specific primers C (5′-CCGAGTCGCGGATCAGCC-3′) and D (5′-CAGTACATTCGGTCATTCACC-3′) using the Expand High Fidelity PCR System (Boehringer) and the hot start PCR program described below. *PPDES6* was cloned into the pCR-Script Amp SK(+) cloning vector (Stratagene), resulting in plasmid p*PPDES6* and sequenced on both strands.

Transformation of P. patens

First the vector pRT101neo was constructed to obtain a *npt* II selection cassette, which could be excised by *Hin*dIII digestion. For this purpose the *npt* II coding region of pRT100neo (Töpfer *et al.*, 1993) was excised with *Hin*dIII (blunted)/*Xho*I and ligated between the CaMV 35S promoter and terminator of pRT101 (Töpfer *et al.*, 1987), which had been digested with *Xba*I (blunted)/*Xho*I. The gene disruption construct resulted from the substitution of a *Sau*I/*Bst*BI fragment in the genomic clone p*PPDES6* by the *npt* II selection cartridge. Subsequently, the disruption construct was digested with *Aat*II and *Hpa*I, resulting in a linear fragment with the *npt* II gene in its centre flanked by genomic sequences of 923 bp and 1159 bp. Fifteen micrograms of this linear DNA were phenol extracted, precipitated and used for the transformation without separation from the vector. PEG-mediated direct DNA transfer into protoplasts was performed as described by Schaefer *et al.* (1991). The regenerated protonemata were selected for 14 days on medium with G418 (50 mg l^{-1}), released for 12 days under non-selective conditions and again grown for 14 days on

selection plates. Well growing plants surviving this selection regime were defined as stable transformants and cultivated for mass production in non-selective liquid medium. The stability of their G418 resistance was tested every 4 weeks by incubating aliquots on selection plates.

DIG-labelling of DNA and RNA

DNA probes were labelled with digoxygenin by PCR (PCR DIG probe synthesis kit; Boehringer). The 5′ ends of the primers for the deletion probe (Del) were located on the *PPDES6* cDNA at position 910 and 1092 (*SauI*/*Bst*BI fragment). The desaturase RNA probe was transcribed by *in vitro* transcription with digoxygenin (Boehringer) from a subclone of the *PPDES6* cDNA containing the last 600 bp of its 3′ end and the *npt* II probe from a subclone coding for the *npt* II.

PCR detection, Southern and Northern blot analysis

Four primers were used in the PCR experiments for the detection of gene targeting events. Primer 1 was derived from the 5′ end of *PPDES6*. Primers 2 and 3 were constructed from the ends of the *npt* II coding region. The sequence of primer 4 (5′-CAGAGACGAATCGTGGCTCC-3′) was derived from the 3′ end of an incomplete cDNA clone, which was identical with *PPDES6* cDNA in the overlapping region, but contained a longer 3′ end. The PCR experiments with these primers were run with a hot start programme of 10 min denaturation at 94°C, addition of the polymerase at 72°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 3 min at 72°C and terminated by 10 min extension at 72°C. Genomic DNA of *P. patens* was extracted with cetyl-trimethyl-ammonium bromide according to Rogers and Bendich (1988). Four micrograms of DNA were digested with the appropriate restriction enzyme, separated on a 0.7% agarose gel by electrophoresis, transferred onto a nylon membrane and hybridized. The final washing steps were performed in 0.5 × SSC with 0.1% SDS at 68°C. The detection was accomplished with a chemiluminescent substrate (CSPD, Boehringer). The Northern blot experiments were performed with total RNA isolated from 14-day-old protonema cultures (RNeasy plant kit, Qiagen, Hilden, Germany). Twenty micrograms of total RNA were separated on a standard formaldehyde gel, blotted onto a nylon membrane and hybridized with RNA probes. The final washing steps were performed in 0.1 × SSC with 0.1% SDS.

Expression in S. cerevisiae

The open reading frame of the *PPDES6* cDNA was cloned behind the galactose-inducible promotor *GAL1* of the yeast expression vector pYES2 (Invitrogen, Leek, Netherlands). For this purpose, a new *Xho*I site was introduced by PCR (32 bp upstream from its deduced translational start at position 319). The entire open reading frame of the desaturase was released with *Hin*dIII (blunted)/*Xho*I and ligated into the *Xba*I (blunted)/*Xho*I sites of the pYES2 vector to yield plasmid pYESΔ6. Its sequence was verified by DNA sequencing. The plasmids pYESΔ6 and pYES2 were transformed into the *Saccharomyces cerevisiae* strain INVSC1 (Invitrogen) by the lithium acetate method (Ausubel *et al.*, 1995). Cells harbouring the plasmids pYES2 and pYESΔ6 were grown in complete minimal drop-out uracil medium (CMdum) containing 2% raffinose as the exclusive carbon source (Ausubel *et al.*, 1995; Kajiwara *et al.*, 1996) and 1% Tergitol NP-40 (w/v; Sigma) for the solubilization of fatty acids (Avery *et al.*, 1996). For expression experiments, the cultures were grown to an optical density (600 nm) of 0.5 in CMdum medium, then supplemented with 2% galactose (w/v) as well as 0.003% of the corresponding fatty acid (w/v; stock solution solubilized in 5% tergitol) and finally grown to saturation for 24 h at 30°C.

Lipid analysis

Lipids were extracted from protonemata and yeast cells by chloroform-methanol extraction (Siebertz *et al.*, 1979) and purified from apolar components by TLC in diethylether. In this solvent all membrane lipids (triacylglycerols were not produced by protonemata) remained at the start. The fatty acid methyl esters (FAME) were obtained by transmethylation of the lipids with 1 N H_2SO_4 in methanol and 2% dimethoxypropane at 80°C for 1 h. The extracted FAME were analysed by gas-liquid chromatography using a capillary column (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 m, 0.32 mm). Their identities were confirmed by comparison with appropriate FAME standards (Sigma). The corresponding fatty acid pyrrolidides were obtained as described elsewhere (Andersson and Holman, 1974) and analysed by GLC-MS on a HP 5989 A instrument (Hewlett-Packard) equipped with an HP-5 column using a temperature gradient 150°C (3 min) → 320°C at 5° min^{-1}. Electron impact (EI) was carried out at 70 eV and chemical ionization mass spectra (CI-MS) were recorded with ammonia as reactant gas (0.1 MPa).

Acknowledgements

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Cloning and Molecular Characterization of the Δ6-Desaturase from Two *Echium* Plant Species: Production of GLA by Heterologous Expression in Yeast and Tobacco

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ABSTRACT: The synthesis of GLA (Δ6,9,12-18:3) is carried out in a number of plant taxa by introducing a double bond at the Δ6 position of its precursor, linoleic acid (Δ9,12-18:2), through a reaction catalyzed by a Δ6-desaturase enzyme. We have cloned genes encoding the Δ6-desaturase (D6DES) from two different Macaronesian *Echium* species, *E. pitardii* and *E. gentianoides* (Boraginaceae), which are characterized by the accumulation of high amounts of GLA in their seeds. The *Echium D6DES* genes encode proteins of 438 amino acids bearing the prototypical cytochrome b_5 domain at the N-terminus. Cladistic analysis of desaturases from higher plants groups the *Echium* D6DES proteins together with other Δ6-desaturases in a different cluster from that of the highly related Δ8-desaturases. Expression analysis carried out in *E. pitardii* shows a positive correlation between the *D6DES* transcript level and GLA accumulation in different tissues of the plant. Although a ubiquitous expression in all organs is observed, the transcript is particularly abundant in developing fruits, whereas a much lower level is present in mature leaves. Functional characterization of the *D6DES* gene from *E. gentianoides* has been achieved by heterologous expression in tobacco plants and in the yeast *Saccharomyces cerevisiae*. In both cases, overexpression of the gene led to the synthesis of GLA. Biotechnological application of these results can be envisaged as an initial step toward the generation of transgenic oleaginous plants producing GLA.

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GLA (Δ6,9,12-18:3) is recognized as an EFA in human nutrition. Its deficiency causes several health disorders, and it has also been claimed that its administration prevents some diseases (1–3). In particular, GLA has been shown to improve skin function in elderly people (4), attenuate body fat accumulation (5), and have a selective tumoricidal action over human gliomas (6,7).

GLA is synthesized from linoleic acid (Δ9,12-18:2, LA) by the activity of the enzyme Δ6-desaturase (D6DES) that introduces a new double bond into the Δ6 carbon (8). The same enzyme is able to introduce a Δ6 desaturation into α-linolenic acid (Δ9,12,15-18:3, ALA), producing octadecatetraenoic acid (Δ6,9,12,15-18:4, OTA) (9). Although present in humans, D6DES activity is apparently too low to provide enough GLA to satisfy body needs. Therefore, GLA has interest as an essential nutrient and as a component of some pharmaceutical products and functional foods.

GLA is currently marketed from seeds of a few plant species, e.g., evening primrose (*Oenothera biennis*), common borage (*Borago officinalis*), and black currant (*Ribes nigrum*). However, current sources have been recognized as inadequate for the continuous demand of an expanding market (10–12), since they are not oil-rich plants, and their agronomic practices are not as well developed as for other crops. Therefore, attempts have been made to find alternative sources (13). In this sense, GLA production from genetically modified organisms, including oilseed plants, has been suggested as a possible alternative (13–16). Initial attempts at overexpression of *D6DES* genes in heterologous systems have emphasized the importance of the gene origin and host organism. For instance, functional expression of the *D6DES* gene from a cyanobacterium was very inefficient when performed in a higher plant, tobacco (15), whereas performance of the *Borago D6DES* was much better in the same host plant (17). Therefore, *D6DES* genes from higher plants seem to be a better choice (instead of those from fungi or cyanobacteria) when used as transgenes in oilseed crops.

A group of endemic Macaronesian plants from the genus *Echium* (Boraginaceae) were recently identified as among the richest sources of GLA found in nature (18). Among them, *E. gentianoides* showed an exceptional GLA content in the seed, 28% of total FA (19), which makes this species an interesting source of the *D6DES* gene. However, *E. gentianoides* is a perennial shrub whose flowering takes about 2–3 yr in its natural habitat, a major problem when a study of gene expression in floral tissues and developing fruits is intended. To overcome this problem, a closely related species, *E. pitardii*, was also used in our studies to perform a molecular characterization of the *D6DES* gene. Although somewhat less efficient in GLA seed accumulation (22%), *E. pitardii* has the advantage of an annual life cycle that is completed in about 4 mon in the laboratory.

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Abbreviations: ALA, α-linolenic acid; CaMV, cauliflower mosaic virus; DIG, digoxigenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IPCR, inverse polymerase chain reaction; LA, linoleic acid; MS, mass spectrometry; OTA, octadecatetraenoic acid; PCR, polymerase chain reaction.

In this work, we obtained the genomic sequences for the Δ6-desaturase genes of *E. gentianoides* (*EGD6DES*) and *E. pitardii* (*EPD6DES*). A molecular characterization of these genes, including the expression pattern analysis, was performed. Functionality of the putative *D6DES* product was proved by heterologous expression of *EGD6DES* in tobacco *calli* and in the yeast *Saccharomyces cerevisiae*. In transgenic tobacco *calli* the synthesis of GLA and OTA was achieved from the endogenous substrates LA and ALA, respectively, whereas GLA production in the yeast took place from exogenously incorporated LA. These results pave the way for the generation of oilseed plants overexpressing the *EGD6DES* gene.

MATERIALS AND METHODS

Plant material. Seeds from *E. gentianoides* Webb ex Coincy and *E. pitardii* A. Chev. were collected in their natural habitats at the Macaronesian island of La Palma (Canary Islands) during the summers of 1999 and 2000. Tobacco plants, *Nicotiana tabacum* var. Wisconsin 38, were used for transformation experiments. All plants were grown at 25°C under controlled conditions in growth cabinets with a 16 h light/8 h dark photoperiod.

Microbial strains. Agrobacterium tumefaciens LBA4404 (20) was used as a vector for plant transformation. The *S. cerevisiae* strain used in this work was Sc340: *MATa ade1 leu2 ura3 his3::*P_{GAL10}*-GAL4-URA3* (21,22). Cloning procedures in *Escherichia coli* were carried out with strain DH5α as the host.

Cloning and sequence analysis of the D6DES *genes.* Cloning of the *D6DES* gene from *EGD6DES* was achieved by polymerase chain reaction (PCR) amplification of a partial sequence, followed by bi-directional genomic walking through inverse PCR (IPCR). Initially, a 550 bp PCR fragment corresponding to amino acid positions 187 to 369 (Fig. 1) was obtained by using the degenerate oligonucleotides BO-1 [5′-AT(A/C)AG(T/C)AT(T/C)GGTTGGTG-GAA(A/G)TGG-3′] and BO-2 [5′-AATCCACC(A/G)TG-(A/G)AACCA(A/G)TCCAT-3′] as primers and genomic DNA from *E. gentianoides* as a template, following standard PCR protocols. The product was cloned into the pGEM-T-Easy® vector (Promega, Madison, WI) and the sequences for several clones were obtained from both strands by the dideoxy method using a PerkinElmer (Foster City, CA) ABI-377 DNA automated sequencer. Two sequences were obtained that were identified as corresponding to the putative Δ6-desaturase and the highly related Δ8-desaturase, based on the comparison to the orthologous genes from *B. officinalis*. From the *D6DES* partial sequence, two nested upstream primers, GE-1 (5′-GAGGTGAGCGAGCTAAACAACTTG-3′) and GE-2 (5′-AACATATTGACCCTAGCGGAACA-3′), and two nested downstream primers, GE-3 (5′-CTCGGTGA-CTGGAATGCAACAAG-3′) and GE-4 (5′-CGGCGAGT-GTTTATGTTGGTCAG-3′), were designed to perform the IPCR essentially as described (23). The DNA was digested with one of the enzymes *Hin*dIII or *Ssp*I and subjected to circularization followed by two nested rounds of PCR amplification. Suitable fragments were analyzed and sequenced as described before. With this approach, we obtained about 2.5 kbp of genomic sequence, comprising 120 bp upstream of the initiation ATG and some 1.1 kbp downstream of the stop codon.

A genomic DNA fragment containing the whole coding sequence (besides 36 and 97 bp of the 5′- and 3′-untranslated regions, respectively) for *EGD6DES* was obtained by PCR amplification using suitable upstream GE-5 (5′-TGGATCACCAAA-CACAGTAGTAAG-3′) and downstream GE-6 (5′-TCCAACA-AGTAGAACCAATGCAAG-3′) primers and a reading-proof polymerase (AccuTaq®, Sigma). The fragment was cloned and sequenced as indicated. Similarly, a whole genomic clone for the *D6DES* gene from *EPD6DES* was obtained by PCR on genomic DNA, using the same GE-5 and GE-6 flanking primers.

Cladistic analysis. Alignment of amino acid sequences for desaturase proteins was done using the program Clustal X v.1.7 (24) (European Molecular Biology Laboratory, Heidelberg, Germany) with the default settings. For the selected sequences in Figure 1, the alignment was visualized using the Boxshade v. 3.21 program (European Molecular Biology Laboratory). The whole alignment output was used to generate a phylogenetic tree (Fig. 2) based on the neighbor-joining algorithm of Saitou and Nei (25) with the following parameters: The whole amino acid sequence of the protein was considered, positions with gaps were not excluded, and distances were not corrected for multiple substitutions. Bootstrap values over 1,000 replicates were also calculated using the same program. The resulting phenogram was drawn using the program TreeView (University of Glasgow, Glasgow, United Kingdom) (26).

Southern and Northern blot analysis. Genomic DNA was isolated from *Echium* seedlings by a cetyltrimethylammonium bromide-based extraction procedure (27). DNA (about 3 μg) was restricted with the appropriate restriction enzymes, separated on a 0.8% agarose gel, and transferred by capillarity onto Hybond™ N+ nylon membranes (Amersham, Buckinghamshire, United Kingdom). Filters were fixed by baking, prehybridized at 42°C for 5 h in the 50% formamide/High SDS buffer recommended by the digoxigenin (DIG) manufacturer (Boehringer-Mannheim, Mannheim, Germany), and hybridized at the same temperature and same buffer solution (stringent conditions), containing the DIG-labeled *EGD6DES* specific probe at 25 ng/mL. High stringency washes were performed twice at 65°C during 15 min in buffer containing 0.5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and 0.1% SDS, and the luminogenic substrate CSPD® (Boehringer-Mannheim) was used for detection following the instructions provided with the DIG detection kit. Images were obtained by exposure of Biomax ML® films (Kodak, Rochester, NY) for 10–25 min and final developing by standard procedures. The *EGD6DES* probe was obtained by random primed labeling from a PCR fragment (Fig. 3) generated with primers GE-4 and GE-6, corresponding to the last 115 amino acids of the protein and about 100 bp of the 3′-untranslated region. Probe specificity was previously confirmed by the absence of cross-hybridization under the same conditions with a highly homologous Δ8-desaturase fragment from *Echium* (not shown).

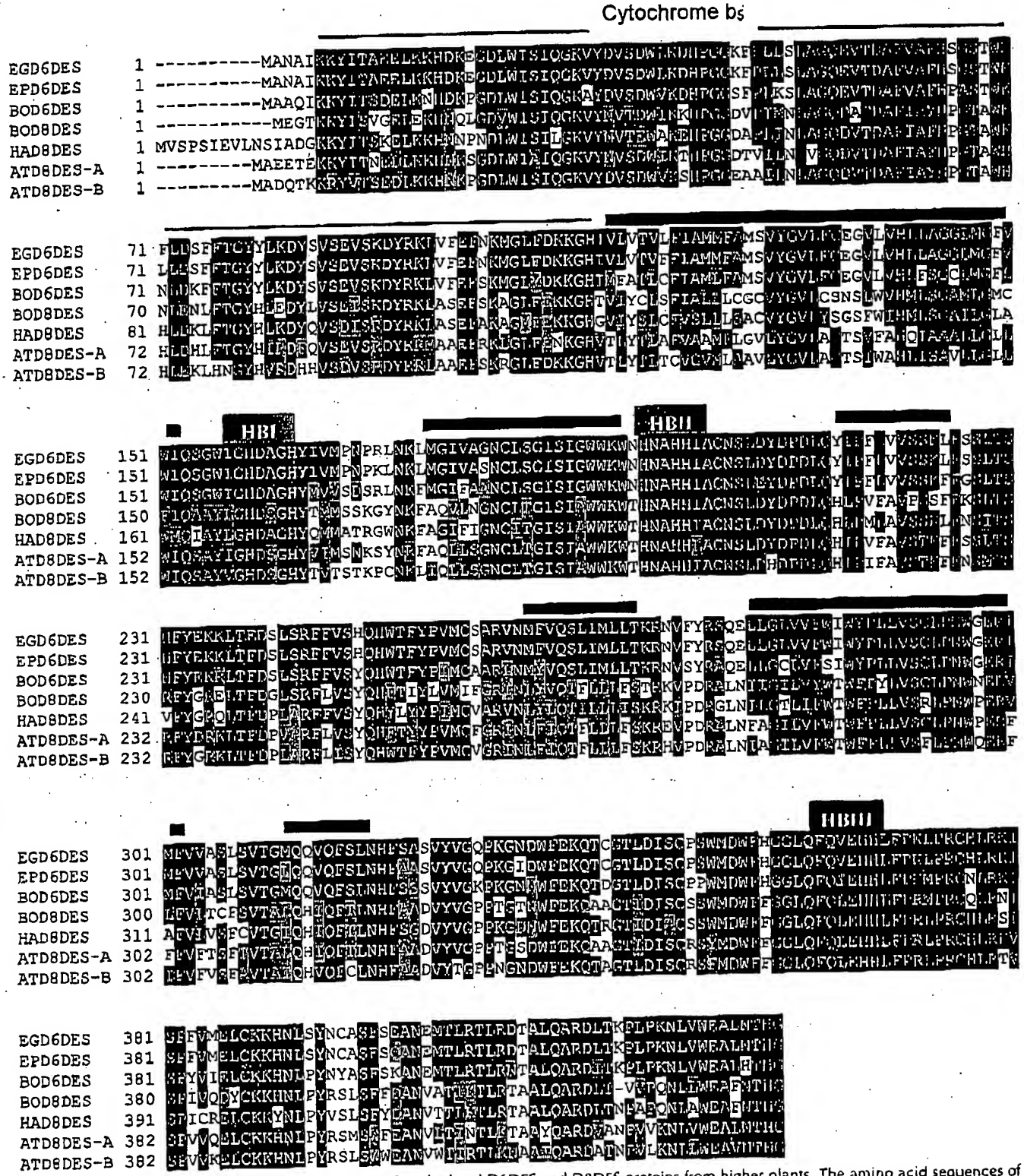

FIG. 1. Sequence comparison between *Echium* D6DES and related D6DES and D8DES proteins from higher plants. The amino acid sequences of -Δ6-desaturases of *E. gentianoides* (EGD6DES, acc. no. AY055117), *E. pitardii* (EPD6DES, AY055118), *Borago officinalis* (BOD6DES, U79010), and Δ8-desaturases from *B. officinalis* (BOD8DES, AF133728), *Helianthus annuus* (HAD8DES, S68358), and *Arabidopsis thaliana* (ATD8DES-A, AAC62885.1; and ATD8DES-B, CAB71088.1) are aligned by using the Clustal X (v1.7) software (European Molecular Biology Laboratory, Heidelberg, Germany). The Boxshade program (European Molecular Biology Laboratory) is used to highlight the homology between protein sequences. Shading is applied when there is agreement for a fraction of sequences above 0.5. Amino acids identical to EGD6DES are enclosed in black boxes and similar residues are in grey boxes. The N-terminal cytochrome-b_5 domain, as well as the position of the three characteristic histidine boxes (HBI to HBIII), are also indicated. Putative transmembrane regions of EPD6DES predicted by the TMPRED software (48) are marked with thick black bars.

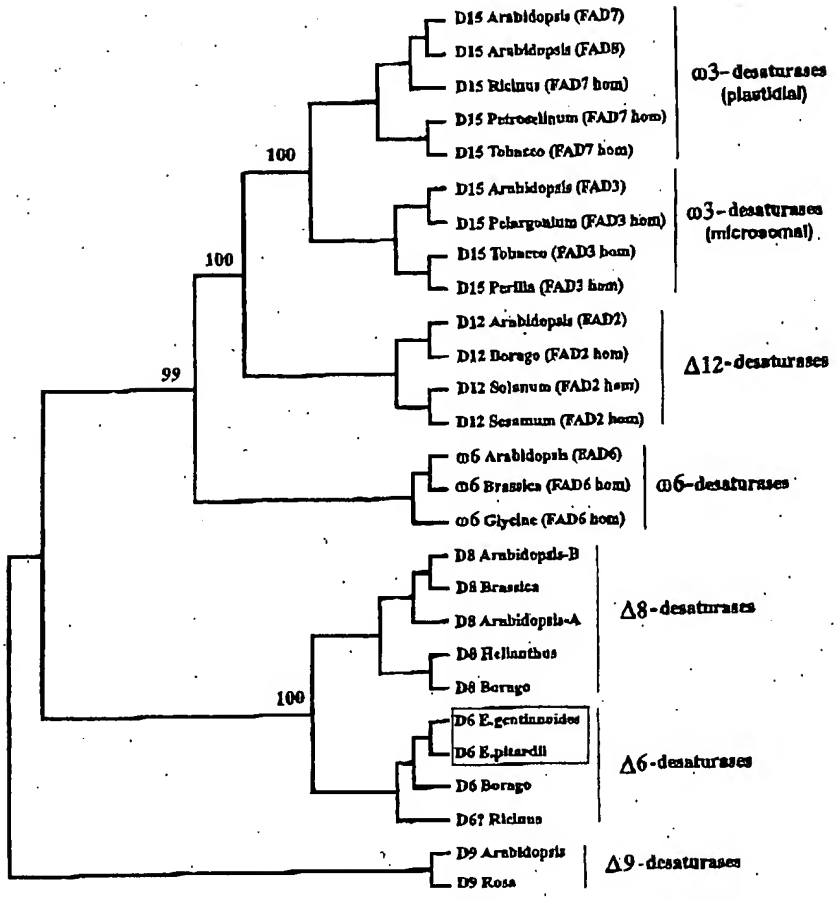

FIG. 2. Neighbor-joining tree illustrating relationships among *Echium* D6DES proteins and other desaturases from dicot species. Amino acid sequences were analyzed as described in the Materials and Methods section. EGD6DES and EPD6DES proteins reported in this paper are indicated enclosed by a square. The tree was rooted using Δ9-desaturases as the outgroup. Bootstrap values are expressed as percentages (over 1000 replicates) at relevant nodes.

Total RNA was extracted from different tissues of *E. pitardii* plants grown to maturity following the method of Chang *et al.* (28). About 5 μg per lane of total RNA was loaded onto an agarose/formaldehyde gel, electrophoretically separated, and transferred to Hybond-N+ membranes. Filters were hybridized at 50°C (stringent conditions) as described for Southern analysis using the same *EGD6DES* specific probe. Stringent washes, accomplished at 68°C, and detection of the DIG labeled probe were as indicated before. As a control, the filters were rehybridized with a 900 bp cDNA probe from tobacco, which encodes part of the cytosolic glyceraldehyde-3-phosphate dehydrogenase gene (29). In this case, hybridization was done in the same conditions, but the final washes were performed at 65°C.

Tobacco transformation and generation of calli. A full DNA genomic fragment encoding the whole EGD6DES protein and part of the 5′ and 3′-untranslated regions was obtained by PCR as indicated before. The fragment was sequenced to check for the absence of PCR mutations and cloned in the sense orientation at the polylinker of the transcriptional fusion vector pJIT60 (30) between a 35S CaMV promoter containing a duplicate enhancer sequence and appropriate termination–polyadenylation signals. The *KpnI-XhoI* "cassette" containing the transcriptional fusion was liberated and cloned into the pBIN19 binary vector (31), and the resulting construct was used to transform the *A. tumefaciens* LBA4404 strain. Tobacco leaf disc transformation was achieved essentially as described by Horsch *et al.* (32). The *calli* were obtained by incubation of tobacco leaf disks in Murashige-Skoog medium containing 30 g/L glucose, 0.1 mg/L 6-benzylamino-purine, 1 mg/L α-naphthalene acetic acid, 100 mg/L kanamycin, 500 mg/L cefotaxime, and 1 mL/L vitamins (stock solution from Sigma). Incubation was performed in petri dishes kept in growth chambers at 25°C under a 12L/12D photoperiod regime.

Yeast transformation. Saccharomyces cerevisiae Sc340 strain was transformed by the procedure of Ito *et al.* (33) with plasmid pYexD6D. This plasmid was constructed by cloning the *E. gentianoides D6DES* gene under control of the *CYC-GAL* hybrid promoter of expression vector pEMBLyex4 (34). Transformant colonies were selected by complementation of the *leu2d* marker. Yeast-transformed clones were grown for about 48 h at 30°C in standard minimal medium supplemented with the auxotrophic requirement of the strain. The

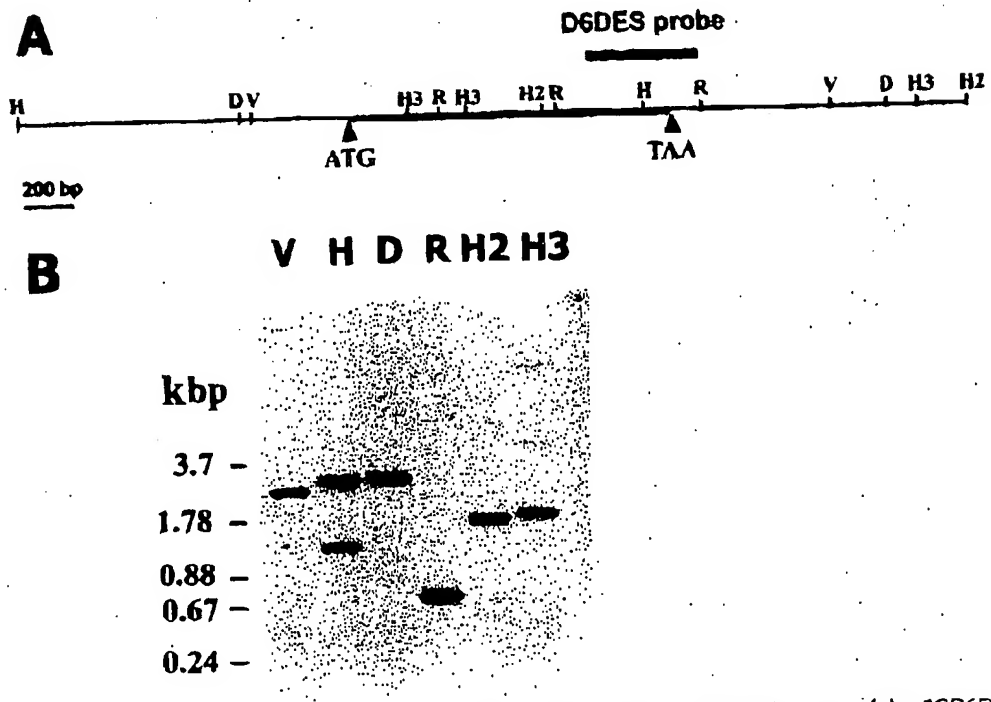

FIG. 3. Genomic structure of the *D6DES* gene of *Echium gentianoides*. (A) Restriction map of the *EGD6DES* genomic sequence reconstructed by inverse polymerase chain reaction. Enzyme symbols are stated below. Position of the *EGD6DES*-specific probe is indicated by a bar. (B) Southern blot analysis of *EGD6DES* in *E. gentianoides*. DNA was digested with *Hind*III (H3), *Hind*II (H2), *Rsa*I (R), *Dra*I (D), *Hae*III (H), or *Vsp*I (V) restriction endonucleases, and hybridization was performed under stringent conditions, as described in the Materials and Methods section, using the *EGD6DES*-specific probe (Fig. 3B). Size markers (kbp) positions are indicated.

cells were collected by centrifugation and transferred to a medium suited for the induction of the *GAL* promoter, which contained 1% yeast extract, 2% peptone, 0.5% glucose, and 1% galactose. The induction medium also contained 25 μM of the GLA precursor LA (final concentration) prepared in Tergitol (1% final concentration). The transformant cells were incubated in the induction medium at 20°C and samples were taken for analysis after 24 and 48 h.

FA analysis. The biomass (tobacco *calli* or yeast) was previously lyophilized. Simultaneous lipid extraction and generation of FAME were performed as described elsewhere (35). For some experiments, fresh tobacco *calli* were directly analyzed following the same method. FA composition was determined by GLC as in (35). GC–MS analysis was carried out using a Varian (Palo Alto, CA) 3400 gas chromatograph–Saturn 3 ion trap mass spectrometer operating at an ionization voltage of 70 eV with a scan range of 60–650 Da. The mass spectra of the relevant peaks were compared to those of standards processed by the same equipment.

RESULTS

Cloning of genes encoding the Δ6-desaturase from Echium *plants.* The complete genomic sequence for the *D6DES* gene of *E. gentianoides* was obtained by PCR amplification of a partial sequence fragment, followed by walking in both directions by IPCR (see the Materials and Methods section). Degenerated primers corresponding to highly conserved motives (ISIGWWKW, and MDWFHGG) of Δ6- and Δ8-desaturases (Fig. 1) were used to amplify from the genomic DNA of *E. gentianoides* a 550 bp PCR fragment that was subsequently cloned in a T-vector. Several clones were sequenced, allowing the identification of putative Δ6- and Δ8-desaturase sequences based on the comparison to the *Borago* orthologues. From the partial *D6DES* sequence, gene-specific primers were designed that allowed the obtaining of several IPCR clones and the assemblage of a 2.5 kbp genomic sequence containing the whole coding region. Finally, genomic fragments comprising the coding sequence of genes from *EGD6DES* and *EPD6DES* were obtained by PCR amplification on genomic DNA using flanking primers derived from the *E. gentianoides* sequence. None of these genes contained intervening sequences showing 98% of identity at the DNA level. The two genes presented very similar open reading frames encoding proteins of the same length, 438 amino acids (Fig. 1). The *Echium* proteins shared a high similarity, with only seven amino acid changes (three of them conservative). Both proteins are highly homologous to Δ6- and Δ8-plant desaturases (Fig. 1). They exhibit the prototypical cytochrome b_5 domain at the N-terminus, as reported for other Δ6- and Δ8-desaturases (36). In addition, three Histidine boxes, the third one conforming to the characteristic consensus QXXHH of these desaturases, are found at the corresponding positions. Putative trans-membrane regions are predicted to occur

(Fig. 1) according to their location as membrane-bound proteins. Protein BLAST search revealed the highest homology to D6DES of *B. officinalis*, sharing 85% of identical residues with EGD6DES. Clustering analysis including representative members of the different desaturase classes shows a clear grouping of the two *Echium* proteins with the D6DES of *Borago* and a still uncharacterized protein from *Ricinus* (acc. no. AF005096). These proteins make up a separate clade (supported by a 100% bootstrap value) to that of the highly related Δ8-desaturases. This provides evidence for the identification of the *Echium* gene products as Δ6-desaturases.

Genomic organization and expression analysis of the Echium *Δ6-desaturase.* Genomic structure and organization of the *D6DES* gene in *Echium* was investigated by Southern-blot analysis. Genomic DNA from *E. gentianoides* was digested with six different restriction enzymes followed by hybridization with a *EGD6DES* specific probe (see the Materials and Methods section) under stringent conditions. Single hybridization bands were obtained with five enzymes that do not cut within the probe (Fig. 3B), thus indicating that the *EGD6DES* gene is represented in the haploid genome by a single copy. Moreover, sizes for the hybridization fragments comprising the coding region are in agreement with the genomic sequence assembled by IPCR walking (Fig. 3A), thus confirming that the *D6DES* genes do not possess introns. Similar results were obtained from Southern analysis in *E. pitardii* (results not shown).

The expression pattern of the *D6DES* was analyzed by Northern blot on total RNA from different tissues of *E. pitardii*. Hybridization with the *EGD6DES* specific probe under stringent conditions resulted in relatively strong hybridization signals for samples corresponding to stems, roots, flowers, and developing fruits, this latter tissue giving the highest intensity. Conversely, the *EPD6DES* RNA was found at a comparative low level in the leaves (Fig. 4A). In the same experiment expression of the housekeeping gene *GAPDH* served as a positive hybridization control. The relative transcript levels (normalized in base to RNA loading) of *EPD6DES* showed a good correlation with the GLA content determined for the different tissues (Fig. 4B). This might be an indication that in *Echium* plants the GLA content in a given tissue might be primarily governed by the steady-state level of the *D6DES* mRNA.

Functional analysis of the Echium *Δ6-desaturase by heterologous expression in tobacco* calli. In order to demonstrate that the *Echium* genes actually encode enzymes with a Δ6-desaturase activity, we have introduced *via Agrobacterium* the complete *EGD6DES* gene under the control of a constitutive 35S CaMV double promoter in tobacco, a plant that does not accumulate GLA. The phospholipid-bound LA, which is

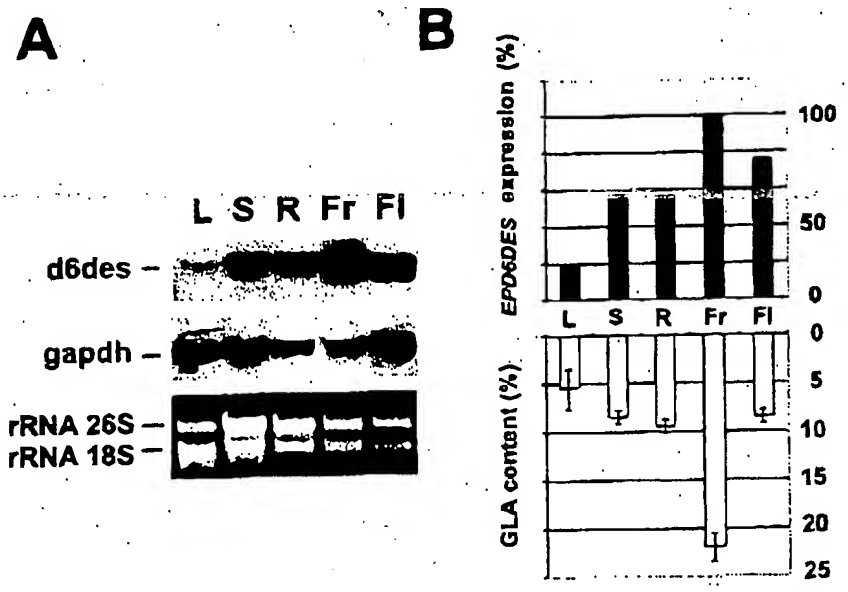

FIG. 4. Expression analysis of the *EPD6DES* gene. (A) Northern experiment of the *D6DES* of *Echium pitardii*. Equivalent amounts of total RNA from leaves (L), stems (S), roots (R), developing fruits (Fr), and developing flowers (F) were hybridized with the *EGD6DES*-specific probe (Fig. 3A) under high-stringency conditions, as indicated in the Materials and Methods section. The expression pattern of the cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a constitutive gene used as a control, is shown below beside the ethidium bromide staining of the gel. The *GAPDH* probe was obtained from tobacco. (B) Comparison of the GLA content and *D6DES* expression in different tissues of *E. pitardii*. Relative *D6DES* expression (above), normalized in base to RNA loading, is expressed as a percentage over the maximum level attained in developing fruits. GLA content is shown (below) as a percentage of total FA. These data were determined in triplicate as described in the Materials and Methods section, and the mean values beside their SE intervals are indicated in the figure.

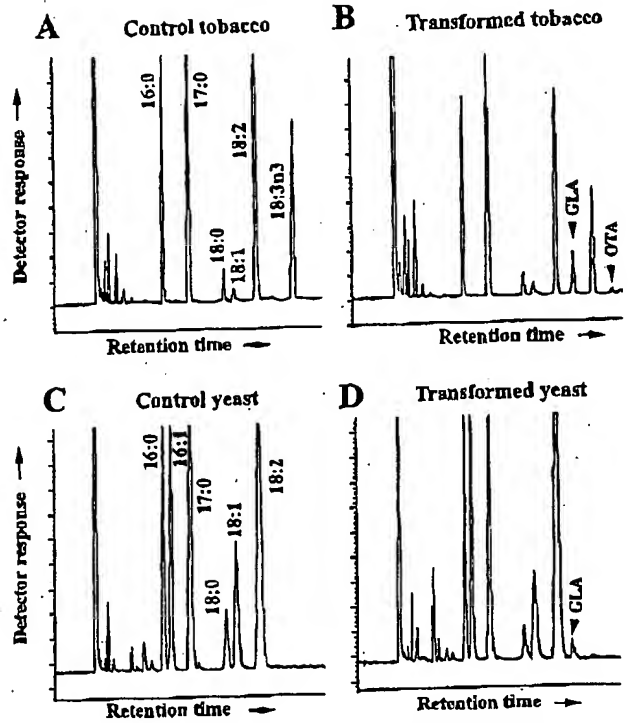

FIG. 5. Identification by GC of GLA in tobacco and yeast transformed with the *Echium D6DES* gene. FAME of total lipids of tobacco *calli* (A,B) and *Saccharomyces cerevisiae* (sc340) grown under inductive conditions in the presence of the linolenic acid substrate (C,D). (A) Control tobacco *calli* transformed with the pBIN19 vector alone. (B) Tobacco *calli* transformed with the *EGD6DES* gene under control of the CaMV constitutive promoter. (C) Control yeast transformed with the pEMBLyex4 vector alone. (D) Yeast transformed with the *EGD6DES* gene under control of the $P_{CYC\text{-}GAL}$ inducible promoter. Positions of the additional peaks (GLA and OTA) are indicated by arrows. Heptadecanoic acid (17:0) is included in the analysis as an internal standard. OTA, octadecatetraenoic acid.

the main substrate for D6DES, is present at a high level in tobacco tissues. Therefore, synthesis of GLA was expected in the transformed plants. To speed-up the assay, *calli* derived from leaf discs were analyzed for FA composition. As a negative control tobacco *calli* transformed with the pBIN19 vector alone was used. A total of 22 *calli* samples representing individual transformation events were screened for both the control and *35S::EGD6DES* constructs. In all transgenic samples harboring *EGD6DES*, the presence of an additional peak with a retention time corresponding to GLA was apparent (Fig. 5B), as compared to the control (Fig. 5A). Further identification of this compound as GLA was achieved by GC–MS using the pure GLA-methyl ester as a standard (data not shown). The mass spectra of the unknown and GLA standard were identical. In this experiment, GLA was found in variable amounts ranging in the different samples from 1.9 to 11.8% on total FA (Table 1). This variation should be attributed to positional effects of the T-DNA insertion in each individual *callus*. The presence of a second additional peak corresponding to the OTA was also observed in a number of transgenic samples (Figs. 5A and 5B). Identification by GC–MS of the new peak was

TABLE 1
FA Composition of Total Lipids from Tobacco *Calli* Transformed with the *EGD6DES* gene

	Control[a]		*35S::EGD6DES*	
FA[b]	Range[c]	Mean[c] ± SEM	Range[c]	Mean[c] ± SEM
16:0	18.0–19.8	19.2 ± 0.06	18.3–20.6	19.2 ± 0.08
18:0	3.2–3 .7	3.4 ± 0.02	3.0–4.0	3.3 ± 0.03
Δ9-18:1	0.8–2.0	1.3 ± 0.03	1.3–2.4	1.8 ± 0.03
LA	35.3–40.6	37.9 ± 0.16	31.6–41.6	35.3 ± 0.31
GLA	—	—	1.9–11.8	6.5 ± 0.27
ALA	17.8–25.6	22.4 ± 0.24	16.4–23.4	18.6 ± 0.21
OTA	—	—	0.0–1.6	0.5 ± 0.06
Total FA	1.3–1.5	1.4 ± 0.01	1.2–1.8	1.5 ± 0.02

[a]Control experiment correspond to *calli* transformed with the empty vector pBIN19.
[b]Contributions of individual FA are expressed as percentage of total FA. Total FA are calculated as percentage of the dry callus weight.
[c]The results are from the analysis of 10 individual *calli*. LA, linoleic acid; ALA, α-linolenic acid; OTA, octadecatetraenoic acid.

achieved by comparison with the OTA standard (data not shown). Again this compound was expected to be produced by the D6DES activity using the endogenous ALA as a substrate. It is noteworthy that, when detected, the amount of OTA was consistently lower than GLA (Table 1).

Functional expression of the Δ6-desaturase from Echium *in the yeast* S. cerevisiae. The complete coding region of *EGD6DES* (see the Materials and Methods section) was cloned in the yeast expression vector pEMBLyex4 under control of the $P_{CYC\text{-}GAL}$ promoter inducible by galactose. The resulting plasmid (pYexD6D) was used to transform *S. cerevisiae* strain Sc340. This strain carries a construction in which the *GAL4* gene under control of the P_{GAL10} promoter is located at the site that interrupts the *HIS3* gene. *GAL4* encoded an enhancer protein that triggers the expression of genes activated by galactose. The GAL4 protein is present in the cell in very low amounts. Therefore, the Sc340 construction amplifies the expression of genes placed under control of *GAL* promoters (21,22). The transformed yeast strains were cultured under inductive conditions in the presence of LA provided as exogenous substrate. This FA is not synthesized by the yeast, but it is efficiently incorporated, reaching about 70% of the total FA in the cell under our experimental conditions. Extracts of yeast cells transformed with the *EGD6DES* gene showed a peak not present in the extracts of control cultures transformed with the vector alone (Figs. 5C and 5D). The retention time in GC experiments coincides with that of GLA, and the identity of the compound was confirmed by GC–MS analysis (not shown). In this case, GLA accumulation reached a maximum of 1.5% of total FA.

DISCUSSION

Functional characterization of the Echium D6DES *gene.* Here we provide evidence for the identification of the gene products EGD6DES and EPD6DES as functional Δ6-desaturases. First, a high similarity of their protein sequences was found with other previously characterized D6DES, mainly *B.*

officinalis, a species belonging to the same family of plants. However, as it has been pointed out (11), sequence similarity among desaturases should be considered cautiously before assigning a particular function to an unknown protein. For example, a sunflower desaturase, showing a high homology to the *Borago* D6DES (BOD6DES), was identified as a Δ8-desaturase active on sphingolipids rather than on glycerolipids (37). This is further supported by directed mutagenesis experiments in which few amino acid changes in a desaturase resulted in drastic alterations in the substrate specificity regarding acyl chain length and desaturation position (38). Nevertheless, clustering analysis of desaturases from higher plants separates with a high reliability the group integrated by BOD6DES and the *Echium* desaturases from that of the Δ8-desaturases, thus indicating that the *Echium* products are likely to be Δ6-desaturases.

To obtain a direct proof for the activity of the putative *Echium* desaturases we overexpressed the *EGD6DES* gene in tobacco *calli*. FA composition of these undifferentiated cells is similar to that of nonphotosynthetic tissues, such as the root, where a higher amount of LA (45%) relative to ALA (26%) is found (39). These compounds were previously shown to act as substrates of D6DES enzymes, giving rise to GLA and OTA, respectively (9,40). Both products were detected in transformed *calli*, thus demonstrating the ability of the *Echium* enzyme to desaturate the Δ6 position of FA containing previous double bonds at Δ9 and Δ12 carbons. These results also indicate that the activity of a single D6DES enzyme is enough to synthesize both GLA and OTA.

The activity of EGD6DES has been further analyzed by heterologous expression in a yeast system. As expected, desaturation of exogenously provided LA was observed in transformed cells. A similar experiment has been carried out with the D6DES from the fungus *Mortierella alpina* (13). In this case Δ6-desaturation of the endogenous 16:1n-7 and 18:1n-9 was observed in transformed yeast that were not supplemented with LA. Accumulation of 16:2n-7 has also been reported by yeast expression of the *Borago* D6DES (41). However, in our experiments we did not detect the accumulation of such compounds. This discrepancy could be attributed to differences in the experimental conditions, or it might reflect a different behavior of the D6DES from those organisms.

Gene structure and expression analysis of the Echium D6DES *gene*. We have shown that the *D6DES* of *Echium* do not contain introns. The same seems to be true for the *Borago D6DES* and the *D8DES* from *Echium* and *Borago*, since partial sequences obtained by genomic PCR also present continuous open reading frames (results not shown). Moreover, the whole coding sequences of the two *D8DES* genes (F2A19.180; At2g46210) present in the *Arabidopsis* genome are also contained within single exons. This is in contrast to the gene structure of D6DES from other organisms such as the moss *Physcomitrella* (42), the worm *Caenorhabditis* (43), and *Homo* (44), where a number of introns interrupt both the desaturase and cytochrome b_5 domains. Although we cannot exclude the existence in *Echium* of an additional version of the *D6DES* gene containing introns, this is not supported by Southern analyses since they do not reveal the presence of further loci. It seems likely therefore that loss of introns of the *D6DES* gene may have occurred during evolution of higher plants, followed, at least in Boraginaceae species, by high divergence or loss of the interrupted gene. Loss of introns has been well documented in a number of instances, with retroposition as the most direct mechanism (revised in 45). The fact that *D8DES* genes are also continuous suggests that the retroposition event took place before the duplication and further divergence of the *D6DES/D8DES* lineages.

We have also studied the expression of the *D6DES* gene in different tissues of *E. pitardii*. High relative levels of D6DES transcript is found in all tissues of the plant except in leaves of mature plants, where it is barely detectable. This correlates with the accumulation of GLA in those tissues, as the synthesis of this compound in the mature leaves is about five times below that of the developing fruits. The lower GLA level in the leaves has also been reported in other plants (46,47), and competition between the D6DES and the ω3-desaturase for the same substrate (LA) to give either GLA or ALA, respectively, was suggested as the cause (9). It is likely that this competition has its origin at the transcriptional level. This correlation suggests that, at least in *Echium*, the steady-state level of the D6DES transcript might be the limiting factor in the accumulation of GLA in the different tissues.

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